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STEM CELLS®
Original Article

LIF/STAT3 Signaling Fails to Maintain Self-Renewal of Human Embryonic Stem Cells

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Key Words. Human embryonic stem cells · Leukemia inhibitory factor · STAT3 · Self-renewal

ABSTRACT

Murine embryonic stem (mES) cells remain undifferentiated in the presence of leukemia inhibitory factor (LIF), and activation of signal transducer and activator of transcription 3 (STAT3) via LIF receptor (LIFR) signaling appears sufficient for maintenance of mES cell pluripotency. Anecdotal and contradictory accounts exist for the action of LIF in the culture of human embryonic stem cells, and the nature of LIF signaling and whether the LIF-STAT3 pathway is conserved in human embryonic stem cells (hESCs) has not been systematically explored. In this study, we show that the LIFRβ and the signaling subunit gp130 are expressed in hESCs and that human LIF can induce STAT3 phosphorylation and nuclear translocation in hESCs. Nevertheless, despite the functional activation of the LIF-STAT3 signaling pathway, human LIF is unable to maintain the pluripotent state of hESCs. Feeder-free culture conditions that maintain hESCs in an undifferentiated state do not show activation of STAT3, suggesting that distinct signaling mechanisms govern the self-renewal of hESCs. *Stem Cells 2004;22:770–778*

INTRODUCTION

Human embryonic stem cells (hESCs) are pluripotent cells derived from the inner cell mass of blastocysts [1]. Previous studies have shown that hESCs can be stably maintained in culture for longer than 1 year and have the capacity to differentiate in vitro and in vivo into cell types from the three germ layers [2, 3]. These characteristics make them an invaluable resource for studies of cell differentiation and development. A major challenge is to determine the conditions that allow convenient and efficient large-scale culture of hESCs and their directed differentiation into therapeutic cells. hESCs are routinely cultured on feeder fibroblasts to maintain their undifferentiated state [1, 2], but feeder-free culture systems have recently been developed [4]. hESCs cultured on Matrigel supplemented by conditioned media from mouse embryonic fibroblasts (MEF-CM) remain positive for markers of the undifferentiated state (e.g., oct-4, hTERT, alkaline phosphatase, TRA-1-60, and SSEA-4), retain the morphology of undifferentiated cells, and retain the potential to differentiate into numerous cell types. This work suggests that one or more

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factors secreted by MEFs are required to maintain hESC self-renewal. Murine embryonic stem (mES) cells can be kept undifferentiated in culture under feeder-free conditions by adding leukemia inhibitor factor (LIF) to the medium [5, 6]. LIF is a cytokine that belongs to the interleukin-6 (IL-6) family, which includes IL-6, oncostatin M, cardiotrophin-1, IL-11, and ciliary neurotrophic factor. The LIF receptor (LIFR) consists of the following two subunits: gp130, which is common to all the cytokines from the IL-6 family, and LIFRβ (or gp190), specific for LIF [7]. Both signal transducer and activator of transcription (STAT) and Ras/mitogen-activated protein kinase pathways are activated downstream of gp130. The activation of STAT3 appears both necessary and sufficient for mES cell self-renewal [8]. Overexpression of a dominant-negative form of STAT3 in mouse embryonic stem (ES) cells inhibits their self-renewal and enhances their differentiation [9], whereas a conditionally active form of STAT3 is sufficient to maintain the undifferentiated state of mouse ES cells [10]. Although the central role of the LIF-STAT3 signaling has been well documented in mES, the function of this pathway has remained unclear in hESCs. While anecdotal reports suggest that LIF is not required for maintenance of hESCs [1, 2], others claim that "LIF helps retain the hES cells in an undifferentiated state" [11]. In this work, we investigated whether LIF was necessary and sufficient for maintaining hESCs in an undifferentiated state and whether pathways activated by LIF in hESCs are functionally conserved between mouse ES and hESCs.

Materials and Methods

Culture of ES Cells

The hESC lines WA09 (H9) and UC06 (HSF-6) were cultured on feeder layers (primary MEFs from Specialty Media [Phillipsburg, NJ]) in the following medium: knockout-Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 20% serum replacement (Knockout SR [Gibco]), 1 mM L-glutamine, 1% nonessential amino acids, 0.1 mM beta-mercaptoethanol, and 4 ng/ml human basic fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ). Cells were passaged every 3 days by dissociation with 0.25% trypsin/1 mM EDTA. hESCs were also maintained on Matrigel basement membrane matrix (BD Biosciences, Bedford, MA) with MEF-conditioned medium supplemented with bFGF (4 ng/ml). The mES cell lines CCE, J1, and E14 were cultured as described [12]. When elimination of the feeder layer was necessary, we harvested the cells with trypsin-EDTA and plated them in WA09 medium for 45 minutes to allow MEFs to adhere. Nonadherent cells were then collected.

Reverse Transcriptase Polymerase Chain Reaction Analysis

RNA was prepared using RNA STAT60 (Tel-test) according to the manufacturer's instructions. RNA (1.5 μg) was reverse transcribed with superscript reverse transcriptase (RT) (Invitrogen). For polymerase chain reaction (PCR), 1 μl of cDNA was used in a 25-μl final volume using 1.25 units of Taq polymerase (Promega, Madison, WI) with the following primers: gp130 forward: 5'-ggagtgctgttctgctttaa-3', gp130 reverse: 5'-actgtgtaccacggtagaat-3', LIFR forward: 5'-ccta acagatggtggagtg-3', LIFR reverse: 5'-gctgatcgagtttccagaac-3', actin forward: 5'-tggcaccacaccttctacaatgagc-3', actin reverse: 5'-gcacagcttctccttaatgtcacgc-3', Rex1 forward: 5'-cagatcctaaacagctcgcagaat-3', Rex1 reverse: 5'-gcgtacgcaaat-taaagtccaga-3', Nanog forward: 5'-actaacatgagtgtggatcc-3', Nanog reverse: 5'-tcatcttcacacgtcttcag-3'. After 25 cycles, 10 μl of PCR product was separated on 1.5% agarose gel.

Immunoblot

hESCs or mES cells were plated on Matrigel or 0.2% gelatin, respectively, on 6-well plates (1×10^6 cells per well). One day later, the cells were washed with phosphate-buffered saline (PBS) and cultured with medium containing 0.1% serum replacement without cytokines. The next day, the cells were stimulated with 0.01 μg/ml murine LIF (mLIF) or human LIF (hLIF) (Chemicon, Temecula, CA) for 10, 20, 30, or 60 minutes, washed with PBS, and harvested with 0.25% trypsin-1 mM EDTA. Protein extracts were prepared by resuspending the cells in lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 10 mM NaF, 1 mM EDTA, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, 1% Nonidet P-40 [NP-40], 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM Na_3VO_4), and proteins were resolved on a 10% SDS polyacrylamide gel. The antibodies used were phospho STAT3 (Tyrosine 705), STAT3 antibody (Cell Signaling, Beverly, MA), phospho STAT3 (serine 727) antibody (Biosource, Camarillo, CA), oct 4 (BD Transduction Laboratories), Jak-2 (Upstate, Lake Placid, NY), and HDJ-2 (Lab Vision Corporation, Fremont, CA).

Assay for Differentiation

hESCs were plated at 1×10^5 on Matrigel or 0.2% gelatin and grown in the presence of MEF-CM, hLIF (10 ng/ml), or medium without hLIF. Expression of TRA-1-60 [13], a specific marker of undifferentiated hESCs, was checked after 8 and 16 days.

Surface-Antigen Expression

For antibody staining, hESCs were dissociated with Trypsin-EDTA. Cells were then stained with the primary antibody TRA-1-60 (provided by Dr. Peter Andrews). Phycoerythrin-conjugated goat anti-mouse IgM was used as secondary anti-

body. Samples were run through a FACScan cytometer (Becton, Dickinson, Franklin Lakes, NJ).

Subcellular Fractionation

Nuclear and cytoplasmic fractions were prepared as previously described [14]. Briefly, cells were harvested in CE buffer (10 mM HEPES [pH 7.6], 60 mM potassium chloride, 1 mM EDTA, 1 mM dithiothreitol) and supplemented with 0.275% NP-40 and protease inhibitors. After centrifugation at 2,000 rpm for 5 minutes at 4°C, the supernatant (cytoplasmic fraction) was stored on ice and the nuclear pellet was washed twice with CE buffer, resuspended in 60 μl of norepinephrine buffer (20 mM Tris [pH 8.0], 420 mM NaCl, 1.5 mM MCl_2, 0.2 mM EDTA, 25% glycerol), and supplemented with protease inhibitors. After 10 minutes on ice, both fractions were centrifuged at 13,000 rpm for 10 minutes at 4°C, and supernatants were transferred to fresh tubes.

Retroviral Transduction of hES with a Constitutively Active Form of STAT3

Murine STAT3C cDNA (provided by Daniel C. Link) was cloned as an *Eco*R1 fragment into the MSCViresGFP (MIG) retroviral vector [15]. Retroviruses were produced by transient cotransfection of 293T cells with the following three plasmids: the viral vector MIG-STAT3C or MIG-empty, the gag-pol genes encoding retroviral packaging plasmid pUMVC3, and a plasmid encoding the vesicular stromatitus virus G protein [16]. Viruses containing supernatants were collected 48 hours after transfection and concentrated by ultracentrifugation. The mES cells and hESCs were transduced with retroviruses by a single-round infection (for 24 hours) at a multiplicity of infection (MOI) of 1, 10, and 50.

Results

Effect of LIF on the Maintenance of hESCs in an Undifferentiated State

We first assessed the potential of hLIF to maintain hESCs in an undifferentiated state. WA09 (H9) cells were grown on Matrigel or gelatin in the presence or absence of hLIF or with a combination of hLIF and bFGF. To assess the state of differentiation, we measured the level of expression of the TRA-1-60 antigen that selectively recognizes undifferentiated hESCs [1, 2]. WA09 cells grown on MEFs or on Matrigel supplemented with MEF-CM maintained consistent and high-level expression of the TRA-1-60 antigen (Fig. 1A). In contrast, when WA09 cells were cultured on gelatin or Matrigel supplemented with hLIF, marked downregulation

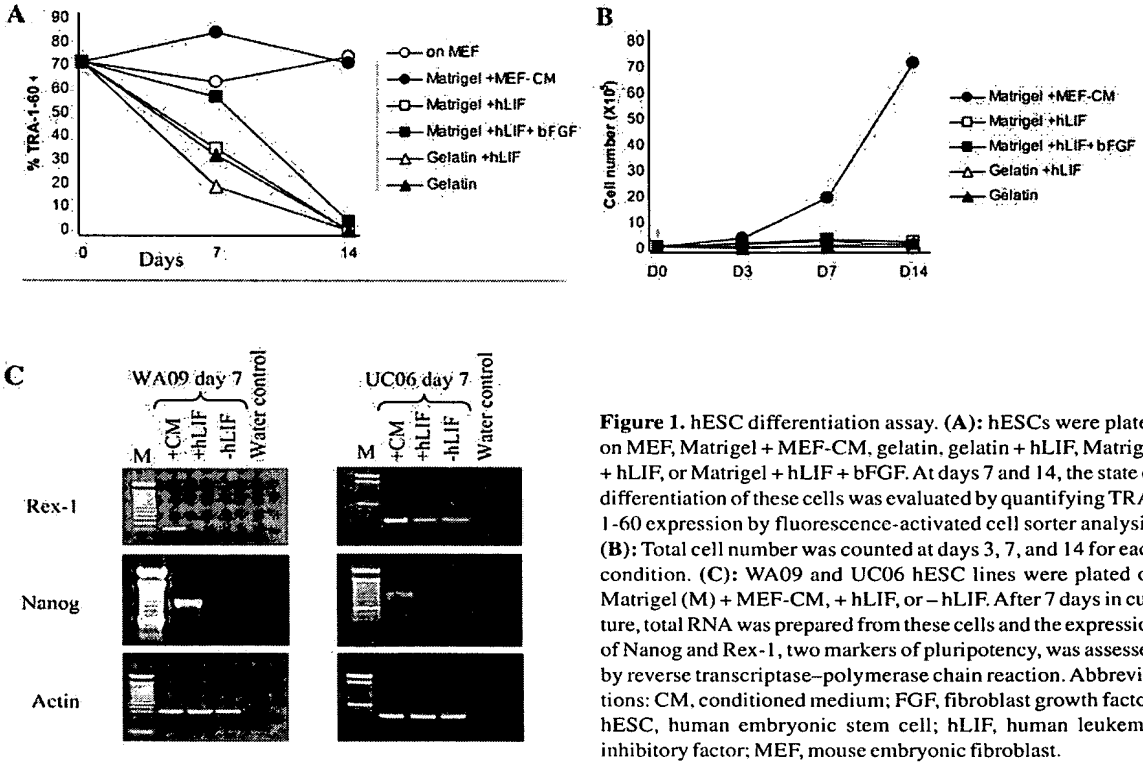

Figure 1. hESC differentiation assay. **(A):** hESCs were plated on MEF, Matrigel + MEF-CM, gelatin, gelatin + hLIF, Matrigel + hLIF, or Matrigel + hLIF + bFGF. At days 7 and 14, the state of differentiation of these cells was evaluated by quantifying TRA-1-60 expression by fluorescence-activated cell sorter analysis. **(B):** Total cell number was counted at days 3, 7, and 14 for each condition. **(C):** WA09 and UC06 hESC lines were plated on Matrigel (M) + MEF-CM, + hLIF, or – hLIF. After 7 days in culture, total RNA was prepared from these cells and the expression of Nanog and Rex-1, two markers of pluripotency, was assessed by reverse transcriptase–polymerase chain reaction. Abbreviations: CM, conditioned medium; FGF, fibroblast growth factor; hESC, human embryonic stem cell; hLIF, human leukemia inhibitory factor; MEF, mouse embryonic fibroblast.

of TRA-1-60 expression was seen after 7 days and was almost complete by 15 days (Fig. 1A). The same pattern of TRA-1-60 downregulation was found for UC06 (HSF-6) cells grown under comparable conditions (data not shown). Downregulation of TRA-1-60 expression in both hESC lines correlated with overt signs of morphological differentiation (not shown). We counted the number of cells for each condition (Fig. 1B) and showed that hESCs grown on Matrigel with MEF-CM expand in culture whereas cells grown on Matrigel and gelatin (with or without LIF) stopped proliferating after 3 to 5 days and assumed a differentiated morphology. We also assayed by RT-PCR the expression of two markers of ES cell pluripotency, Nanog and Rex1 [17–19]. Both Nanog and Rex-1 are expressed at high levels in WA09 and UC06 cells grown for 7 days on Matrigel and MEF-CM (Fig. 1C). Corroborating the downregulation of TRA-1-60 expression and morphologic differentiation, Nanog and Rex-1 expression likewise decreased when the cells were cultured on Matrigel alone, with or without hLIF. Unlike WA09 cells, UC06 still expressed low levels of Rex-1 after 7 days with or without hLIF. This difference is likely attributable to the lower growth rate of UC06 compared with WA09 and therefore slower kinetics of differentiation. These data confirmed that hLIF is not sufficient to maintain hES in the pluripotent, undifferentiated state.

Expression of LIFR and gp130 in hESCs

Given the lack of response of hESCs to hLIF, we investigated whether the components of LIF receptor signaling were expressed on the cells. The receptor for the cytokine LIF consists of two subunits, the LIF receptor LIFRβ (gp190) and the signal transducer gp130. After depleting the WA09 and UC06 hESCs of contaminating MEFs, we prepared total RNA, generated cDNA by RT, and performed PCR amplification using primers for LIFR or gp130. Both LIFRβ and gp130 were detected in WA09 and UC06 cells (Figs. 2A, B). No amplification was obtained from MEF cDNA, which confirms that the primers were specific for the human sequence. As positive controls, cDNA from K562 and TF1, two human cell lines known to express the LIF receptor, were amplified. Furthermore, expression of gp130 protein was confirmed by immunoblotting WA09 cells, mES, and MEFs with an antibody that recognizes both mouse and human gp130 (Fig. 2C). These data indicate that the components of the LIF receptor complex are expressed in hESCs.

Activation of STAT3 Phosphorylation by LIF

In mES cells, the binding of LIF to its receptor triggers the activation of associated Jak tyrosine kinases, which in turn phosphorylate STAT3 [20]. Therefore, we asked whether LIF could induce STAT3 phosphorylation in hESCs. Both mES cells (on gelatin) and hESCs (on Matrigel) were cultured for 12 hours without LIF in 0.1% serum replacement before stimulation with either mLIF or hLIF. We prepared whole-cell extracts from these cells and assayed for STAT3 phosphorylation using an antibody recognizing phosphotyrosine at position Y705, a residue critical for STAT3 dimer-

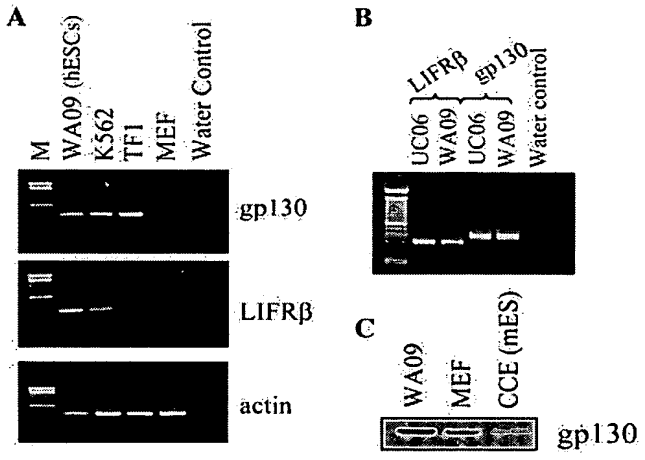

Figure 2. Expression of gp130 and LIFRβ in hESCs. **(A):** Total RNA was reverse transcribed, and the cDNA was subjected to PCR using primers for gp130, LIFRβ, and actin (as a loading control). **(B):** Total RNA from WA09 and UC06 human embryonic stem cell lines was reverse transcribed, and gp130 and LIFR were amplified by PCR. **(C):** Total cell extracts from hESCs, MEF, and mES cells were analyzed by immunoblotting using an antibody directed against murine and human gp130. Abbreviations: hESCs, human embryonic stem cells; M, Matrigel; MEF, mouse embryonic fibroblast; mES, murine embryonic stem; PCR, polymerase chain reaction.

ization and nuclear translocation [21]. mLIF induced Y705 STAT3 phosphorylation in mES cells (Fig. 3A). The signal can be detected after 10 minutes, reaches a peak at 20 minutes, and decreases after 30 minutes, in concordance with previous reports [22, 23]. In contrast, no STAT3 Y705 phosphorylation was observed in hESCs in response to mLIF, although STAT3 was expressed (Fig. 3A). We repeated the same experiment with hLIF, and phosphorylation of STAT3 on Y705 was detected in both mES and hESCs (Fig. 3B). These results show that hLIF is able to induce STAT3 phosphorylation in hESCs, whereas mLIF fails to activate hLIF receptor signaling.

STAT3 contains a second phosphorylation site on Serine 727 that is critical for optimal induction of STAT3 transcriptional activity [24]. Therefore, we analyzed STAT3 phosphorylation at S727 upon LIF addition to mES and hESCs. As anticipated, S727 was phosphorylated upon addition of mLIF to mES cells (Fig. 3C). In hESCs, we could detect a basal level of S727 phosphorylation in the absence of LIF and a slight increase of S727 phosphorylation after addition of hLIF Fig. 3C). Taken together, these results suggest that the transcription factor STAT3 can be phosphorylated at both Y705 and S727 by LIF receptor occupancy in hESCs.

Activation of STAT3 by MEF-CM

Because MEF-conditioned medium supplemented with FGF (4 ng/ml) can sustain hESC self-renewal on Matrigel, we asked whether this condition was accompanied by STAT3 phosphorylation. After 12 hours on Matrigel with 0.1% serum replacement, hESCs were stimulated with MEF-CM + bFGF (4 ng/ml) for 15, 30, and 60 minutes. Total protein extracts were prepared from these cells, and STAT3 phosphorylation was assessed by immunoblotting with antibodies directed against phosphotyrosine 705 and phosphoserine 727. The combination of MEF-CM + bFGF did not induce Y705 STAT3 phosphorylation in hESCs, whereas phosphorylation is induced by hLIF (Fig. 4). In contrast, S727 STAT3 phosphorylation is modestly increased after addition of MEF-CM + bFGF (Fig. 4). These data demonstrate that conditions critical for maintaining hESCs in an undifferentiated state (MEF-CM + bFGF) are not associated with STAT3 phosphorylation on the critical residue Y705.

STAT3 Subcellular Localization

Phosphorylation of Y705 is known to promote dimerization of STAT3 and translocation to the nucleus, where it functions in transcriptional activation [21]. We examined STAT3 subcellular localization in mES cells (CCE) and hESCs (WA09) after acute addition of LIF, as well as in mES and hESCs cultured under conditions that maintain the undifferentiated state. Cytosolic and nuclear fractions were prepared from

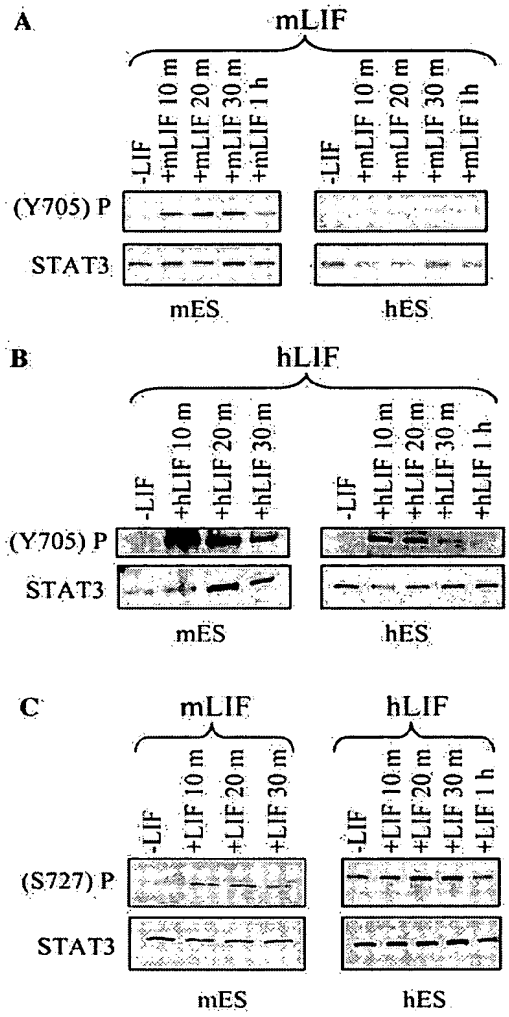

Figure 3. STAT3 phosphorylation after stimulation of mES and hES with murine or human LIF. mES and hESCs were factor-deprived for 12 hours and then stimulated with mLIF (10 ng/ml) **(A)** or human LIF (10 ng/ml) **(B)** for indicated times. Immunoblots of whole-cell extracts were then probed with an antibody directed against phosphotyrosine 705 on STAT3 (Y705 P-STAT3) or total STAT3. **(C)**: Immunoblots of whole-cell extracts prepared from mES and hESCs stimulated with mLIF and hLIF, respectively, for indicated times were probed with an antibody directed against phosphoserine 727 on STAT3 (S727 P-STAT). Abbreviations: hESC, human embryonic stem cell; hLIF, human leukemia inhibitory factor; LIF, leukemia inhibitory factor; mES, murine embryonic stem; mLIF, murine leukemia inhibitory factor; STAT3, signal transducer and activator of transcription 3.

WA09 and CCE cells treated for 10 minutes with hLIF (10 ng/μl) and mLIF (10 ng/μl), respectively. LIF induced the nuclear translocation of STAT3 in both WA09 and CCE cells (Fig. 5A). Moreover, using an antibody against phosphotyrosine 705, we confirmed that the phosphorylated form of

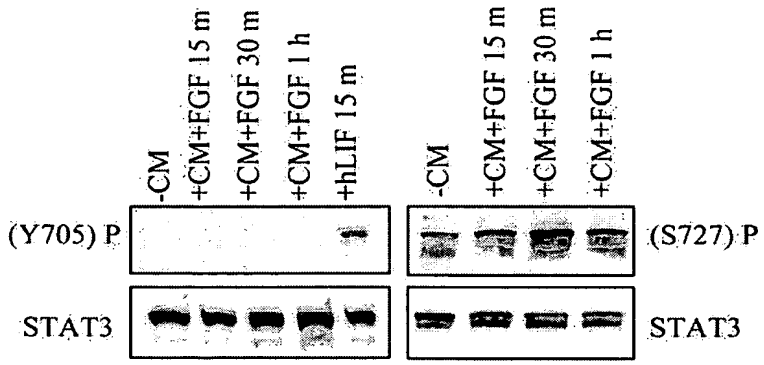

Figure 4. STAT3 phosphorylation after stimulation of hESCs with MEF-CM + bFGF. hESCs were plated on Matrigel. After 12 hours of factor deprivation, cells were stimulated with MEF-CM + bFGF (4 ng/ml). Immunoblots of whole-cell extracts were probed with antibodies directed against phosphotyrosine 705 and phosphoserine 727 on STAT3 or total STAT3. Abbreviations: bFGF, basic fibroblast growth factor; CM, conditioned medium; hESC, human embryonic stem cell; MEF, mouse embryonic fibroblast; STAT3, signal transducer and activator of transcription 3.

STAT3 is found exclusively in the nucleus (Fig. 5A). Effective subcellular fractionation was verified by the detection of the transcription factor Oct-4 protein within the nuclear fractions and the cytoplasmic Jak-2 or HDJ-2 proteins within the cytosolic fractions (Fig. 5A). We determined the localization of STAT3 in undifferentiated mES cells maintained in log-phase growth in the presence of LIF and undifferentiated hESCs grown on Matrigel with MEF-CM. Using an antibody to detect total protein, STAT3 was found predominantly in the cytoplasm of mES cells; however, using the more specific antibody directed against phosphotyrosine 705, the modification critical for nuclear translocation [21], we indeed detected phosphorylated STAT3 in the nuclear fraction (Fig. 5B). In contrast, phosphorylated STAT3 was not found in the nuclear extracts from hESCs cultured under conditions that maintain self-renewal. Taken together, these results indicate that nuclear localization of STAT3 is not associated with conditions that maintain hESCs self-renewal and apparently is not required for maintenance of hESCs in the undifferentiated state.

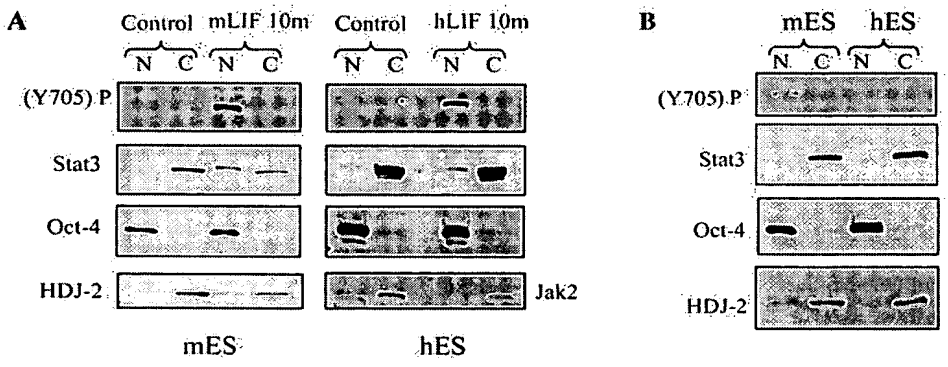

Figure 5. Subcellular localization of STAT3. **(A):** Nuclear (N) and cytoplasmic (C) fractions were prepared from mES cells and hESCs cultured without LIF (control) or 10 minutes after addition of mLIF or hLIF (10 ng/ml), respectively. **(B):** Nuclear and cytoplasmic fractions were prepared from undifferentiated mES cells maintained in log-phase growth in the presence of mLIF and undifferentiated hESCs grown on Matrigel with MEF-CM. STAT3 localization was determined by immunoblotting with antibodies directed against phosphotyrosine 705 on STAT3 and total STAT3. Immunoblotting to detect the nuclear transcription factor Oct-4 was used to validate the nuclear fractionation, whereas detection of the cytoplasmic proteins Jak-2 and HDJ-2 was used as controls for the cytosolic fractionation. Abbreviations: CM, conditioned medium; hESC, human embryonic stem cell; hLIF, human leukemia inhibitory factor; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblast; mES, murine embryonic stem; mLIF, murine leukemia inhibitory factor; STAT3, signal transducer and activator of transcription 3.

Expression of STAT3C in ES Cells

Although our results show that STAT3 activation by LIF appears insufficient to maintain self-renewal of ES cells, we asked whether a constitutively activated allele of STAT3, which has been shown to maintain self-renewal of mES cells, might likewise sustain hESC self-renewal. STAT3C is a mutant form of STAT3 with a substitution of two cysteine residues in the SH2 domain that spontaneously dimerizes, translocates to the nucleus, and activates transcription [25]. We transduced mES and hESCs with MIG-empty or MIG-STAT3C retroviruses at increasing MOIs. Both vectors, MIG-empty and MIG-STAT3C, confer green florescent protein (GFP) fluorescence in infected cells. Although most mES cells remain GFP$^+$ 15 days after infection with MIG-STAT3C at MOI 100 (>75% of infected cells; Fig. 6), the percentage of WA09 cells infected with MIG-STAT3C decreased dramatically from 48% at day 2 to 4% at day 15. We attempted to isolate the low numbers of GFP$^+$ cells by flow cytometry to select for clones that maintain STAT3C expression and self-renewal. However, these attempts failed repeatedly, because growth of STAT3C-expressing hESCs apparently could not be sustained. The level of GFP$^+$ cells infected with MIG-empty remained constant for both mES cells and hESCs. We likewise attempted to express a conditionally activated fusion protein of STAT3 and the estrogen receptor that had been shown previously to support the self-renewal of mES cells [10] but again failed to obtain stable lines of hESCs that remained undifferentiated. Our experience suggests that constitutive activation of STAT3 is not sufficient to maintain hESCs in the undifferentiated state and may be associated with enhanced differentiation or apoptosis.

Discussion

Several studies have shown that LIF-induced STAT3 signaling plays a central role in the maintenance of mES cell pluripotency and self-renewal [5, 6], but the consequences of LIF treatment of hESCs have remained largely undocumented. Some publications have concluded a lack of response of hESCs to LIF [1, 2], whereas others have reported a beneficial effect from adding LIF to the culture medium [11]. Given these anecdotal accounts, significant uncertainty remains regarding whether the LIF-STAT3 signaling pathway is expressed and functionally conserved in hESCs. In this study we show that the receptor and signaling components of the pathway (LIFRβ and gp130) are indeed expressed in two hES cell lines (WA09/H9 and UC06/HSF6)

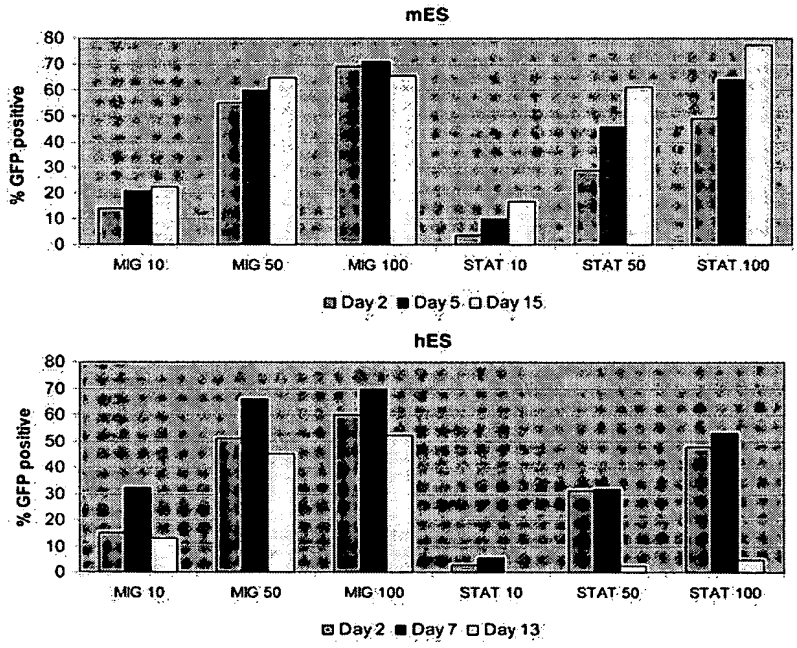

Figure 6. Maintenance or loss of mES and hES cells after retroviral transduction with a constitutively activated form of STAT3. mES and hES cells were infected with MIG-STAT3C at a multiplicity of infection of 10, 50, or 100 or with a control vector (MIG-empty). The percentage of GFP-positive cells was quantified by fluorescence-activated cell sorter after 2, 5, and 15 days for mES (top) and after 2, 7, and 13 days for hES (bottom). Abbreviations: GFP, green fluorescent protein; hES, human embryonic stem; mES, murine embryonic stem; MIG, MSCViresGFP; STAT3, signal transducer and activator of transcription 3.

and that exposure of hLIF to hESCs induces characteristic tyrosine and serine phosphorylation of STAT3 and translocation to the nucleus. However, despite the apparently intact nature of the signaling pathway in hESCs, LIF cannot sustain self-renewal in the absence of differentiation and therefore does not play a comparable role in mouse ES cells and hESCs.

Why hES and mouse ES cells should differ in their mechanisms of self-renewal remains an interesting question. One principal function of LIF in murine development is to enable embryonic diapause [26], the temporary arrest of blastocyst development in lactating female mice that affords optimal timing of multiple and repetitive pregnancies in this highly fecund species. LIF acts as an antidifferentiation factor for cells of the inner cell mass, which can then be propagated as ES cells in what is arguably a fortuitous artifact of cell culture. Because human embryos are not susceptible to diapause, it is perhaps not surprising that blastocyst-derived ES cell lines from the human fail to respond similarly to human LIF, which nonetheless plays critical functions in the hematopoietic, reproductive, endocrine, and central nervous systems [27]. Although by nomenclature, hESCs and mouse ES cells represent a comparable in vitro facsimile of the inner cell mass, the differential response to LIF together with the fact that hESCs but not mouse ES cells show trophoblastic potential [28, 29] argue that hESCs may derive from a more developmentally primitive embryonic cell type that responds to different soluble and cell-associated factors. Because conditioned media from several murine and human cells can indeed sustain hESCs in an undifferentiated state, important self-renewal or antidifferentiation factors remain to be identified.

Our results confirm previous observations about the lack of cross-reactivity of murine and human forms of LIF for the LIF receptor [30]. Indeed, despite high sequence homology between hLIF and mouse LIF (78% amino acid identity) and between the human and mouse LIF receptors (76% identity), mLIF shows a species-restricted binding activity. In contrast, hLIF binds both mouse LIF and hLIF receptor and even shows a higher affinity to the mouse receptor than does mouse LIF itself [31]. In response to LIF cytokine stimulation, the LIF receptor common signaling subunit gp130 activates janus kinases to phosphorylate the transcription factor STAT3 on tyrosine residue 705. Y705 phosphorylation induces STAT3 dimerization, STAT3 translocation to the nucleus, and subsequent DNA binding [21]. The transcriptional activity of STAT3 is also regulated through phosphorylation of serine 727, because an alanine-727 mutant shows reduced transcription factor activity [24]. Both sites must be phosphorylated to achieve the full transcriptional activity of STAT3. In this report, we demonstrated that hLIF could stimulate phosphorylation of both STAT3 Y705 and S727 in hESCs. We also demonstrated that STAT3 translocates to the nucleus after addition of hLIF, and we have detected activation of STAT3 target genes after LIF stimulation (not shown). However, despite apparent functional activation of the STAT3 pathway, hESCs will differentiate despite the presence of hLIF. These results suggest that STAT3 activation is not sufficient to maintain hESCs in an undifferentiated state. This conclusion is additionally strengthened by our failure to isolate self-renewing hESC lines after transduction with either a conditional or a constitutive allele of STAT3. In using a conditionally active form of STAT3 to demonstrate the sufficiency of STAT3 signaling, Matsuda et al. [10] reported that there was a threshold of STAT3 activity required to maintain self-renewal of mES cells, and thus one might argue that hLIF does not achieve the required threshold of STAT3 activation in hESCs. However, we think this is unlikely, because even with methods that yield high gene transduction efficiency, our attempts to express a constitutively activated form of STAT3 in hESCs failed. Indeed, our collective experience suggests increased differentiation or induction of apoptosis in STAT3-expressing hESCs. Finally, we investigated the status of STAT3 activation in hESCs grown on Matrigel with MEF-CM and bFGF, conditions known to maintain these cells in a pristine and undifferentiated state. We found that STAT3 was not phosphorylated on Y705 and could detect only a modest modification at S727. Because Y705 phosphorylation provides the essential prerequisite for dimerization, nuclear translocation, and biological activity, these results suggest that STAT3 is not activated in hESCs cultured under maintenance conditions. Corroborating these observations, we showed that STAT3 was not found in the nucleus of hESCs grown in maintenance media, whereas it is transiently localized in the nucleus of mES cells cultured with LIF.

The work presented here documents the lack of participation of the LIF-STAT3 signaling pathway in the maintenance mechanisms of self-renewal of hESCs. Extensive efforts are underway to unveil the pathways and factors required for propagating ES cells without differentiation, because such agents will be profoundly useful for enhanced ES cell culture.

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Long-term expansion of human functional epidermal precursor cells: promotion of extensive amplification by low TGF-β1 concentrations

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Summary

We have previously introduced the concept of high proliferative potential-quiescent (HPP-Q) cells to refer to primitive human hematopoietic progenitors, on which transforming growth factor-β1 (TGF-β1) exerts a pleiotropic effect. TGF-β1 confers to these slow-dividing cells a mitogenic receptor^{low} phenotype and maintains immature properties by preventing differentiation and apoptosis. However, the effect of TGF-β1 on long-term expansion has not yet been clearly demonstrated. Here, we describe the characterization of a human skin keratinocyte subpopulation, highly enriched for primitive epidermal precursors, on the basis of high adhesion capacity (Adh^{+++}) and low expression of the epidermal growth factor receptor (Adh^{+++}EGF-R^{low}). In our standard culture condition without feeder cells, the mean estimated output for cells from an unfractionated population of primary foreskin keratinocytes was 10^7-10^8, increasing to 10^{12}-10^{13} in cultures initiated with selected Adh^{+++}EGF-R^{low} precursors. Characterization of these cells revealed a hitherto unknown property of TGF-β1: its addition at a very low concentration (10 pg/ml) in long-term cultures induces a very significant additional increase of expansion. In this optimized system, outputs obtained in cultures initiated with Adh^{+++}EGF-R^{low} cells repeatedly reached 10^{16}-10^{17} (~60 population doublings, ~4×10^{18} keratinocytes produced per clonogenic cell present in the initial population). At the molecular level, this effect is associated with an increase in Smad1, Smad2 and Smad3 phosphorylation and an increase in α6 and β1 integrin expression. No such effect could be observed on mature keratinocytes with low adhesion capacity (Adh$^{-/+}$). We finally demonstrated that the progeny of Adh^{+++}EGF-R^{low} precursors after long-term expansion is still capable of generating a pluristratified epidermis in a model for skin reconstruction. In conclusion, after further characterizing the phenotype of primitive epidermal precursors, we demonstrated a new function of TGF-β1, which is to promote undifferentiated keratinocyte amplification.

Key words: Human epidermal precursor, Expansion, Feeder layer-free culture, TGF-β1, High proliferative potential, Reconstructed epidermis, EGF-R

Introduction

Two major obstacles to the study of adult somatic stem cells are the paucity of specific selection markers and our current inability to understand fully the controls of stemness and to exploit the capacity for stem cell self-renewal. In two previous issues of *J. Cell Sci.* (Fortunel et al., 1998; Batard et al., 2000), we have introduced the working model of high proliferative potential-quiescent (HPP-Q) cells to refer to primitive human hematopoietic progenitor cells, on which transforming growth factor-β1 (TGF-β1) exerts an important regulatory role. According to this model, (1) TGF-β1 maintains these cells in a quiescent or slow-cycling state, in part by downmodulating various cytokine receptors, resulting in a mitogenic receptor^{low} phenotype, and thus providing a tool to select this subpopulation (Fortunel et al., 1998); and (2) TGF-β1 may also participate in the control of hematopoietic stem/progenitor cell immaturity. This second function is suggested by the fact that TGF-β1 maintains a high level of the cell-surface expression of hematopoietic cell immaturity markers, such as CD34, throughout successive divisions (Batard et al., 2000). However, because hematopoietic stem and progenitor cells spontaneously differentiate into several lineages and do not remain as a homogenous population in culture, it appeared complex to study the effect of TGF-β1 on long-term self-renewal in this system. As presented in this report, we found that the human epidermal precursor cell compartment represents a unique model to analyze the effect of TGF-β1 on long-term self-renewal of functional undifferentiated somatic cells.

Most studies performed to identify cell-surface markers expressed by primitive keratinocytes have focused on molecules involved in cell adhesion. It has been reported that early keratinocytes of the basal layer of the epidermis express the α2β1 and α3β1 integrins (Peltonen et al., 1989; Carter et

al., 1990). A high expression level of the β1 integrin chain (CD29) has been associated with the high plating efficiency of primitive human keratinocytes in culture (Jones and Watt, 1993; Jones et al., 1995), and with epidermal stem cell functional properties in a murine xenograft model (Jones et al., 1995). More recently, keratinocytes with the greatest proliferative capacity have been shown to express a high level of the α6 integrin chain (CD49f) and, by contrast, a low to undetectable level of the transferrin receptor (CD71) (Li et al., 1998).

In this study, we have first investigated the possibility of isolating a cell subpopulation that was enriched for primitive epidermal precursors with high proliferative potential and maintaining a durable capacity to generate a pluristratified epidermis throughout expansion. This was achieved by selecting cells presenting a mitogenic receptor^{low} cell-surface phenotype, as we previously described for primitive hematopoietic cells in the HPP-Q working model. This selection has been performed on the basis of the cell-surface expression level of the epidermal growth factor receptor (EGF-R), which has largely been described to exert a mitogenic effect on keratinocytes (Cook et al., 1991). On the basis of our data suggesting that TGF-β1 could participate in the control of hematopoietic progenitor cell immaturity, we then explored the capacity of TGF-β1 to control the cell cycling and long-term expansion of immature keratinocytes. The effect of extremely low, yet physiological, concentrations of TGF-β1 (10-30 pg/ml) was analyzed, revealing a hitherto undescribed property of TGF-β1. At these low concentrations, TGF-β1 efficiently promotes the long-term expansion of undifferentiated epidermal precursor cells in a feeder layer-free culture condition.

Materials and Methods

Isolation of primary basal keratinocytes

Human neonatal foreskins obtained at circumcision provided the input material. Samples were first treated with gentamicin (Invitrogen, Paisley, UK). Epithelial sheets were separated from the derma after an overnight incubation with dispase (Boehringer, Mannheim, Germany) at 4°C, followed by 45 minute incubation at 37°C. Basal keratinocytes were isolated by trypsinization (Boehringer) for 15 minutes and fractionated into Adh$^{-/+}$ and Adh^{+++} populations. Samples were deposited on a plastic surface coated with type I collagen (Sigma) and incubated for 12 minutes at 37°C. The Adh$^{-/+}$ cell population were keratinocytes with low adhesion capacity that did not attach to type I collagen within 12 minutes, and the Adh^{+++} population were keratinocytes with high adhesion capacity that remained attached to the substrate after washing. A 12 minute adhesion period was sufficient to obtain a significant enrichment in clonogenic keratinocytes.

Immunofluorescence staining and cell sorting by flow cytometry

For the detection of cell-surface EGF-R, keratinocytes were first incubated for 15 minutes with rat γ-globulins (Jackson ImmunoResearch Laboratories, West Grove, PA), and then for 30 minutes with a non-conjugated monoclonal anti-human EGF-R mouse IgG_{2b} (EGFR1 clone; Dako, Glostrup, Denmark) or an isotypic control from the same species (mouse IgG_{2b}) (Immunotech, Marseille, France). Samples were washed twice and then incubated for 30 minutes with a rat anti-mouse IgG_{2a+b}-PE antibody (Becton Dickinson, San Jose, CA). Analyses and cell sorting were performed using a Vantage Fluorescence Activated Cell Sorter (FACS) (Becton Dickinson).

Cell-cycle and immuno-phenotypic analyses by laser scanning cytometry

For cell-cycle analysis, keratinocytes were plated on glass slides and grown without exogenous TGF-β1 until formation of multicellular clones. TGF-β1 (R&D Systems, Abingdon, UK) was then added to the medium at concentrations ranging from 10 to 3000 pg/ml. Samples were processed 24 hours later for cell-cycle analyses. Cells were fixed and permeabilized in acetone for 30 minutes at –20°C. They were then treated with DNase-free RNase A (Boehringer) for 20 minutes at 37°C, and incubated for at least 15 minutes at room temperature with 20 mg/ml propidium iodide (Sigma). The distribution of the keratinocytes into the G_0/G_1 and $S+G_2/M$ phases of the cell cycle was analyzed in each condition using a Laser Scanning Cytometer (LSC) (CompuCyte, Cambridge, MA). Acquisition and data analysis were performed using Wincyte software (CompuCyte). For the analysis of α6 and β1 integrin expression, keratinocytes from cultures initiated with Adh^{+++}EGF-R$^{-/+}$ cells, previously cultured with or without 10 pg/ml TGF-β1 during four successive passages, were plated on glass slides and grown for three additional days in the same culture conditions. Cells were then fixed in methanol for 5 minutes at –20°C. Prior to α6 or β1 integrin immunostaining, cells were washed in PBS containing 0.2% BSA (PBS/BSA), then incubated respectively with rat or mouse γ-globulins (Jackson ImmunoResearch Laboratories) for 15 minutes, and then with a FITC-conjugated monoclonal anti-human integrin α6 chain (CD49f) rat IgG_{2a} (GoH3 clone; Pharmingen, San Diego, CA), or a FITC-conjugated monoclonal anti-human integrin β1 chain (CD29) mouse IgG_{2a} (K20 clone; Immunotech) for 30 minutes. For Smad phosphorylation studies, keratinocytes from cultures initiated with Adh^{+++}EGF-R$^{-/+}$ cells were plated on glass slides and grown for 72 hours with or without 10 pg/ml TGF-β1. After fixation in methanol for 5 minutes at –20°C, samples were washed twice in PBS/BSA, and then successively incubated for 15 minutes with irrelevant chicken Ig (Jackson ImmunoResearch Laboratories), for 30 minutes with non-conjugated polyclonal rabbit anti-human phosphorylated (p)-Smad1 (Ser463/Ser465) antibodies (sc-12353-R; Santa Cruz Biotechnology, Santa Cruz, CA) or non-conjugated polyclonal rabbit anti-human p-Smad2 and 3 (Ser433/Ser435) antibodies (sc-11769-R; Santa Cruz Biotechnology), and then for 30 minutes with Alexa Fluor488-conjugated chicken anti-rabbit IgG (Molecular Probes, Eugene, OR). Appropriate negative controls were used to determine background signals. The percentage of positive cells, as well as the median value of fluorescence (arbitrary units, a.u.) measured at the level of the population, were evaluated in each culture condition by LSC.

Long-term expansion assays

Cultures were carried out on plastic substrates (25 cm^2 Falcon) in a serum-free medium containing 100 pg/ml EGF, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.4% (v/v) bovine pituitary extract (KGM Bullet Kit; BioWhittaker, Clonetics, San Diego, CA). Cultures were initiated with selected populations of keratinocytes plated at 2400 cells/cm^2. After reaching no more than 70-80% confluence, expanded keratinocytes were detached by trypsinization (Boehringer), counted and replated at 2400 cells/cm^2. Cultures were continually passaged until the growth capacity of the cells was exhausted. Culture medium supplemented or not with the specified concentrations of recombinant human TGF-β1 (R&D Systems) was completely renewed three times per week. The cumulated total cell outputs were calculated assuming that all the cells from the previous passage had been replated. For each cell population and growth factor condition studied, cultures were performed in quadruplicate. At each step, cell viability was evaluated

by trypan blue exclusion. Statistical analyses were performed using the Student's *t* test.

Reconstructed epidermis

Substrate

De-epidermized human dermis (DED) was prepared according to the technique described by Régnier et al. (Régnier et al., 1981). Briefly, split-thickness human skin, obtained at plastic surgery, was floated in magnesium/calcium-free phosphate-buffered saline at 37°C for 10 days. Thereafter, the epidermis was separated from the dermis. Dermal cells were killed by serial freezing and thawing, and the cell-free dermis was stored at –20°C until use.

Reconstruction of an epidermis

Human foreskin keratinocytes cultured as described previously were seeded at different passages onto the DED and cultured for 6 days in Dulbecco's modified Eagle medium/Ham F12 (Invitrogen), containing 10% fetal calf serum (Invitrogen), 10 ng/ml EGF (BD Biosciences, USA), 0.4 μg/ml hydrocortisone (Sigma), 10^{-6} M isoproterenol (Sigma), 5 μg/ml transferrin (Sigma), 2×10^{-9} M triiodothyronine (Sigma), 1.8×10^{-4} M adenine (Sigma) and 5 μg/ml insulin (Sigma). Thereafter, the cultures were raised to the air-liquid interface, and were continued in the absence of isoproterenol, transferrin, triiothyronine and adenine. Histological examination of the reconstructed epidermis was performed after 7 days of culture.

Results and Discussion

Using a mitogenic receptor^{low} cell-surface phenotype, as described for primitive hematopoietic cells (Sansilvestri et al., 1995; Fortunel et al., 1998; Fortunel et al., 2000c), we demonstrated that primitive and highly proliferative human epidermal precursor cells can be isolated. Selection of these epidermal precursor cells was achieved by two successive enrichement steps. Then, through further functional characterization of this human epidermal precursor cell subpopulation, we found that a low physiological concentration of TGF-β1 is able to promote long-term expansion of undifferentiated keratinocytes in a feeder layer-free culture system.

First enrichment step: selection of keratinocytes with high adhesion capacity (Adh^{+++})

An initial enrichment step was based on the knowledge that maturing basal keratinocytes lose their capacity to adhere to extracellular matrix components (Adams and Watt, 1990). The basis of this method is provided by the work of Jones and Watt, who demonstrated that the most primitive keratinocytes with characteristics of stem cells (high colony-forming efficiency and long-term proliferative potentiel) adhered most rapidly to type IV collagen, whereas later keratinocyte populations (transit amplifying cells and post-mitotic differentiated keratinocytes) adhered more slowly (Jones and Watt, 1993). Cells with the highest adhesion capacity (Adh^{+++}) were selected here on a substrate coated with type I collagen, thus eliminating most of the post-mitotic mature keratinocytes. Morphological observation of the two cell populations obtained after adhesion-based separation indicated that the selected Adh^{+++} population is homogenously composed of small-sized undifferentiated cells of less than 12 μm in diameter, whereas the non-selected Adh$^{-/+}$ population is largely heterogenous, mainly composed of differentiating keratinocytes increasing in size. The Adh^{+++} population represented only 10.4% of the total keratinocytes obtained from neonatal foreskins, but was significantly enriched with clonogenic cells that effectively contribute to culture initiation. In a short-term assay, 2.3% of selected Adh^{+++} keratinocytes possessed a clone-forming ability, whereas only 0.2% of the Adh$^{-/+}$ keratinocytes were clonogenic (n>10 independent samples). This is in agreement with a previous study showing an inverse correlation between the size of keratinocytes and their clonogenic potential (Barrandon et al., 1985).

Comparison of the long-term proliferative potential of these populations confirmed that Adh^{+++} keratinocytes were more primitive than Adh$^{-/+}$ keratinocytes (Fig. 1A). The estimated output for one plated Adh^{+++} keratinocyte was at least 100-fold higher than that of unfractionated keratinocytes. By contrast, Adh$^{-/+}$ keratinocytes expressed only a limited proliferative potential, giving rise to ~500-fold fewer cells than a similar number of unfractionated keratinocytes. In the typical experiment shown in Fig. 1A, mean estimated outputs from one plated keratinocyte were 10^9-10^{10} for Adh^{+++} cells, 10^6-10^7 for unfractionated cells, and 10^3-10^4 for Adh$^{-/+}$ cells, corresponding respectively to 31-32 population doublings (PDs), 23-24 PDs, and 12-13 PDs (unfractionated versus Adh^{+++} or versus Adh$^{-/+}$ cells; P<0.01, n>5 experiments). It has been reported that, in post-confluent sheets of cultured human keratinocytes, a process of autoregulation adjusts the frequency of primitive cells independently of their initial frequency in the culture (Jones et al., 1995). The results presented in Fig. 1A show that an initial enrichment in primitive cells at the onset of culture results in an increased cumulated expansion throughout several successive passages, which on the contrary suggests no significant autoregulation of primitive cell frequency. This difference might be explained by the fact that, in the long-term culture experiments presented here, cells were systematically passaged before reaching confluence in order to limit, as far as possible, any regulation of cell fate linked to contact inhibition and homeostasis.

Second enrichment step: sorting of Adh^{+++} epidermal precursor cells presenting a low level of EGF-R expression (Adh^{+++}EGF-R^{low})

In a second enrichment step, we separated by FACS the Adh^{+++} population into four subpopulations of equal size, with increasing levels of cell-surface expression of EGF-R from Adh^{+++}EGF-R$^{-/+}$ to Adh^{+++}EGF-R^{++++} (sorting gates are shown in Fig. 1B). Keratinocytes with the greatest proliferative potential were mostly included in the Adh^{+++}EGF-R$^{-/+}$ subpopulation (~20% of Adh^{+++} cells, ~2% of total keratinocytes). Adh^{+++}EGF-R$^{-/+}$ (Adh^{+++}EGF-R^{low}) cells provided the highest cumulative expansion in long-term cultures compared with Adh^{+++}EGF-R^{++++} (Adh^{+++}EGF-R^{high}) cells (P<0.01, n=5 experiments). In the representative experiment shown in Fig. 1C, the mean output from one plated Adh^{+++}EGF-R$^{-/+}$ keratinocyte was 10^{12}-10^{13} (41-42 PDs, 13 successive passages). Adh^{+++}EGF-R^{++} and Adh^{+++}EGF-R^{+++} keratinocytes promoted a less-efficient

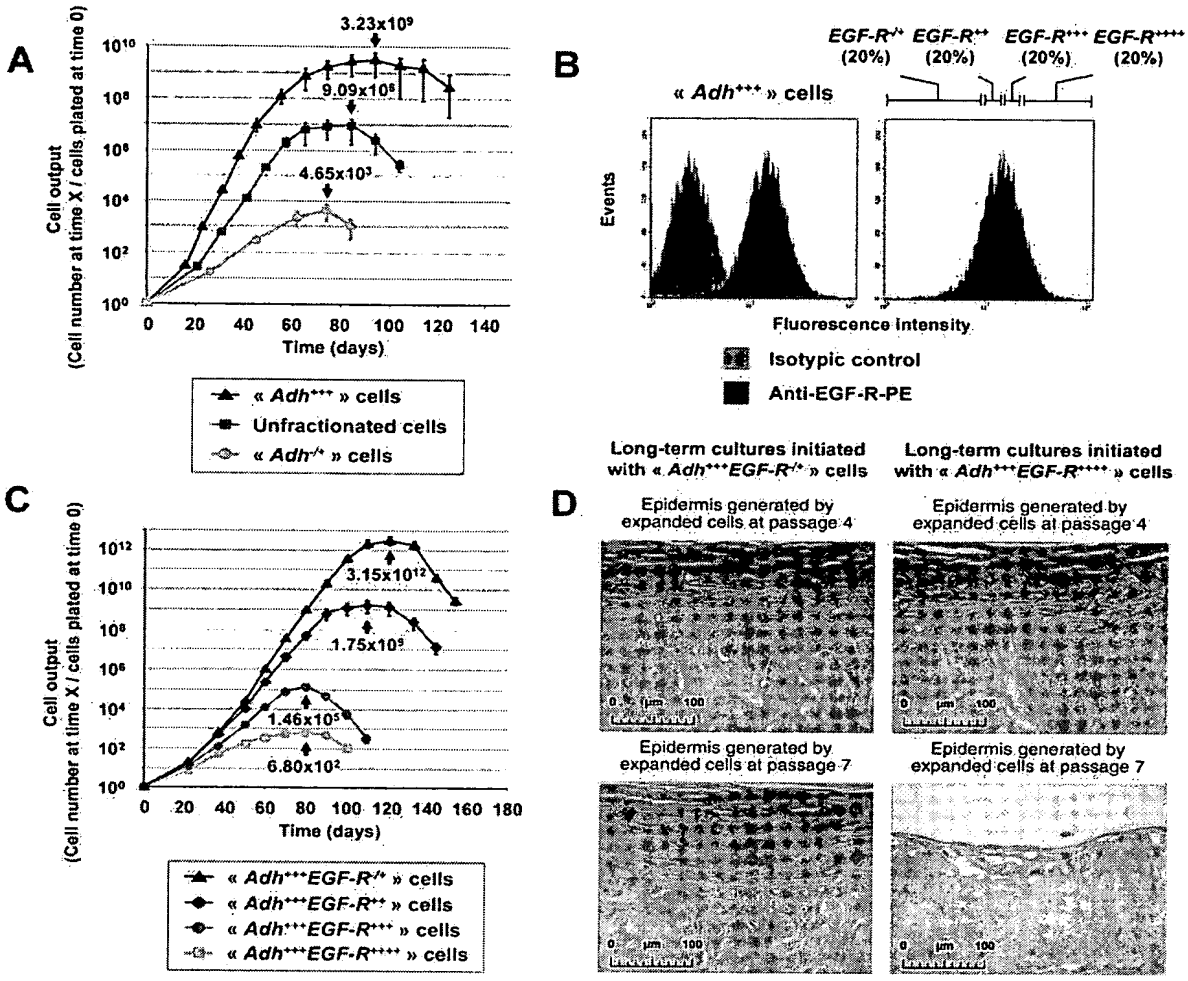

Fig. 1. Characterization of the $Adh^{+++}EGF\text{-}R^{-/+}$ epidermal stem cell subpopulation. (A) Basal keratinocytes were separated on the basis of their adhesion properties. The cell population designated as $Adh^{-/+}$ is composed of keratinocytes with low adhesion capacity, and the Adh^{+++} population is composed of keratinocytes with high adhesion capacity. Cells of each population were studied for their long-term expansion potential. Expansion curves are expressed as a cumulated cell output. Data represent means±s.d. of four replicate cultures from one typical experiment. (B) Cells of the Adh^{+++} population were labeled to analyze their level of EGF-R cell-surface expression by flow cytometry. Sorting gates were defined to isolate four subpopulations: the $Adh^{+++}EGF\text{-}R^{-/+}$ subpopulation contains the 20% of the Adh^{+++} keratinocytes presenting the lowest level of EGF-R expression; the $Adh^{+++}EGF\text{-}R^{++++}$ subpopulation contains the 20% of the Adh^{+++} keratinocytes presenting the highest level of EGF-R expression; the $Adh^{+++}EGF\text{-}R^{++}$ and $Adh^{+++}EGF\text{-}R^{+++}$ subpopulations each contained the 20% of the Adh^{+++} keratinocytes presenting intermediate levels of EGF-R expression. (C) The long-term proliferative potential of $Adh^{+++}EGF\text{-}R^{-/+}$, $Adh^{+++}EGF\text{-}R^{++}$, $Adh^{+++}EGF\text{-}R^{+++}$ and $Adh^{+++}EGF\text{-}R^{++++}$ keratinocytes were compared. Data represent means±s.d. of four replicate cultures from one typical experiment. (D) Capacity of cell subpopulations, sorted according to the level of cell-surface EGF-R expression, to generate a reconstructed epidermis. Selected keratinocytes of the $Adh^{+++}EGF\text{-}R^{-/+}$ and $Adh^{+++}EGF\text{-}R^{++++}$ subpopulations were expanded in defined culture conditions, and then seeded on to a dermal substrate at an early passage (p4) and a late passage (p7) to evaluate their capacity to produce a pluristratified epidermis. Histological preparations shown are from one typical experiment.

expansion: cell outputs of 10^9-10^{10} and 10^5-10^6 (respectively 30-31 and 17-18 PDs, 12 and 9 successive passages). The $Adh^{+++}EGF\text{-}R^{++++}$ subpopulation showed the lowest proliferative capacity with a cell output of 10^2-10^3 (9-10 PDs, 8 successive passages).

Cell sorting based on the $EGF\text{-}R^{low}$ phenotype, as in the HPP-Q working model, is shown here to be effective for selection, within the Adh^{+++} population, of the primitive keratinocytes possessing the greatest expansion potential in long-term culture. It is important to note that EGF-R is not a specific marker of the epidermal stem cell compartment. This tyrosine kinase receptor is widely expressed in the basal and suprabasal layers of the epidermis, regulating not only primitive epidermal cell cycling, but also commitment and terminal differentiation of later keratinocytes (Peus et al., 1998). The starting population used here to sort $EGF\text{-}R^{low}$

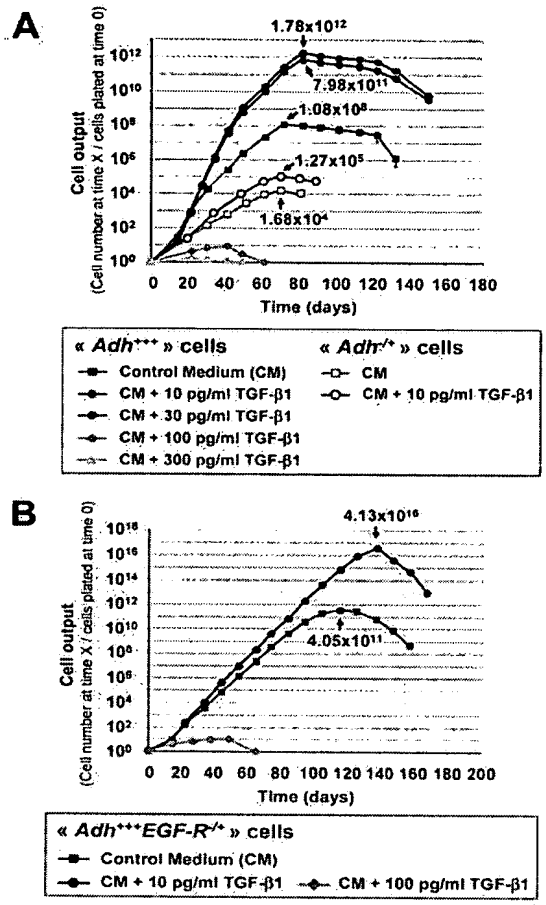

Fig. 2. Dose-response effect of TGF-β1 on the proliferation and expansion of keratinocytes. (A) Long-term cultures were initiated with keratinocytes of the Adh^{+++} or $Adh^{-/+}$ populations, and maintained with or without addition of exogenous TGF-β1 at a concentration of 10, 30, 100 or 300 pg/ml throughout the culture period, as specified. Results are expressed as a cumulated cell output. (B) Long-term cultures were initiated with sorted keratinocytes of the $Adh^{+++}EGF\text{-}R^{-/+}$ subpopulation, and maintained in the presence or absence of exogenous TGF-β1 at a concentration of 10 or 100 pg/ml throughout the culture period. Results are expressed as a cumulated cell output. Data represent means±s.d. of four replicate cultures from one typical experiment. (C) Long-term cultures were initiated with sorted keratinocytes of the $Adh^{+++}EGF\text{-}R^{-/+}$ subpopulation and maintained in the presence or absence of TGF-β1 at the concentration that promotes self-renewal of immature keratinocytes (10 pg/ml). Typical morphology of the cells obtained in these two conditions after 85 and 95 days of culture is illustrated in Fig. 2C.

epidermal precursors consisted of Adh^{+++} cells and not of unfractionated keratinocytes. Indeed, it appeared more appropriate to work on a cell population depleted in more mature keratinocyte populations, in which the level of EGF-R cell-surface expression is not related to stem cell cycling.

Capacity of primitive $Adh^{+++}EGF\text{-}R^{low}$ epidermal precursors to generate a pluristratified epidermis

Another feature of $Adh^{+++}EGF\text{-}R^{-/+}$ ($Adh^{+++}EGF\text{-}R^{low}$) keratinocytes that was evaluated was their organogenic potential. Subpopulations of keratinocytes ($Adh^{+++}EGF\text{-}R^{high}$ or $Adh^{+++}EGF\text{-}R^{low}$) were passaged up to seven times and then seeded onto a dermal substrate. At early passages (p4), keratinocytes initially expressing either a high or a low level of EGF-R were able to form an epidermis (Fig. 1D). In both cases, the characteristic epidermal differentiation pattern was observed: (1) a basal layer containing polygonal cells oriented perpendicular to the underlying dermis; (2) 3-4 layers of spinous cells; (3) 4-6 layers of granular cells characterized by the presence of keratohyalin granules; and (4) anucleated, flattened cornified cells forming a compact stratum corneum. At later passages (p7), only keratinocytes displaying the highest proliferative capacity, namely the $Adh^{+++}EGF\text{-}R^{low}$ subpopulation, were still able to form an epidermis (Fig. 1D), demonstrating a greater capacity of this subpopulation to maintain their organogenic potential throughout in vitro expansion.

Promotion of epidermal precursor cell amplification by low TGF-β1 concentrations

The cytokine TGF-β1 has been described to have a pleiotropic effect on hematopoietic $CD34^+$ stem and progenitor cells by either maintaining them in quiescence (Fortunel et al., 1998; Hatzfeld et al., 1991; Fortunel et al., 2000a; Fortunel et al., 2000b), or by preventing differentiation and apoptosis (Batard et al., 2000; Pierelli et al., 2000). However, the effect of TGF-β1 on long-term expansion of progenitor cells has not yet been demonstrated in a mammalian system. The isolation and characterization of the $Adh^{+++}EGF\text{-}R^{low}$ keratinocyte epidermal precursors revealed a novel function of TGF-β1. It appears to act as a long-term expansion-promoting factor for human primitive keratinocytes. Whereas the concentrations previously used are 10-1000-fold higher, our results demonstrated that, when present at extremely low but physiological concentrations (10-30 pg/ml), TGF-β1 efficiently increased primitive keratinocyte expansion.

In long-term cultures initiated with Adh^{+++} keratinocytes (Fig. 2A shows one typical experiment), the mean estimated

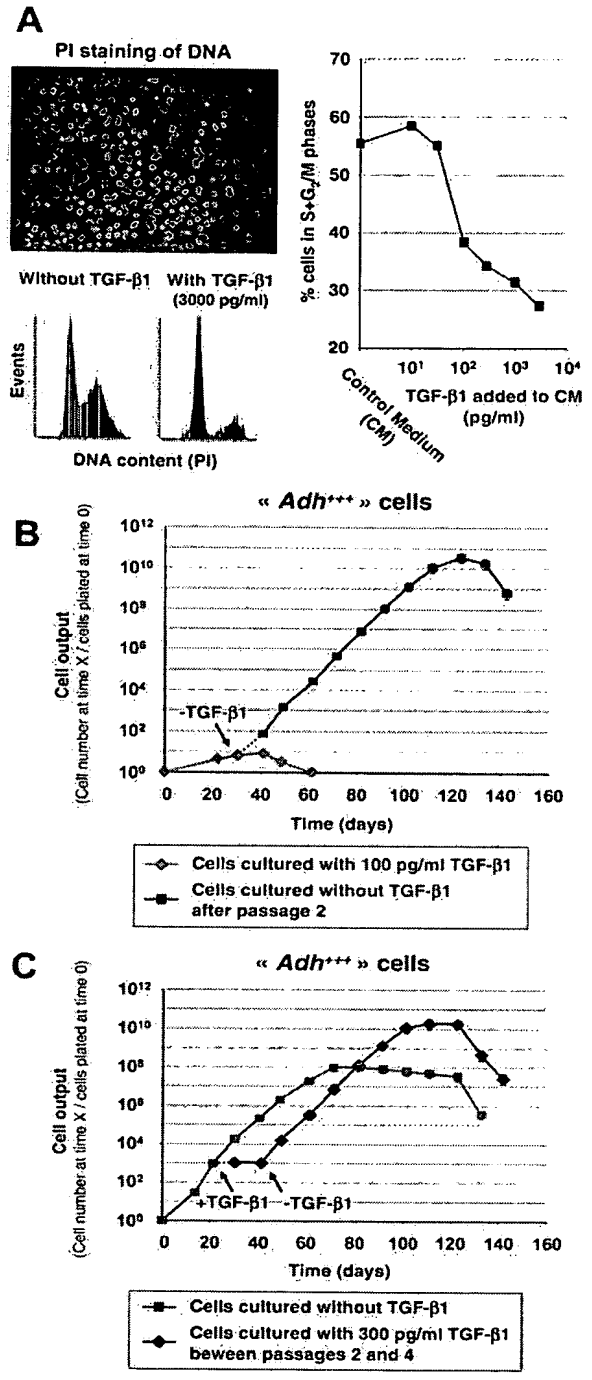

Fig. 3. Reversibility of the inhibitory effect of high TGF-β1 concentrations on keratinocyte cell cycling. (A) Cells of the Adh^{+++} population initially grown on slides without TGF-β1 were cultured for 24 hours with this factor added to the medium at a concentration of 0, 10, 30, 100, 300, 1000 or 3000 pg/ml. Samples were then processed for cell-cycle analysis by Laser Scanning Cytometry (LSC). Data from one typical experiment are shown (image of fluorescent nuclear DNA staining and cell-cycle analyses). (B) Long-term cultures were initiated with keratinocytes of the Adh^{+++} population and maintained in the presence of 100 pg/ml exogenous TGF-β1 up to day 30. Half of the cultures were continued from day 30 without addition of TGF-β1 and the rest were continued in the presence of 100 pg/ml exogenous TGF-β1. (C) Long-term cultures were initiated with keratinocytes of the Adh^{+++} population and maintained up to day 21 without addition of TGF-β1. Half of the cultures were continued from day 21 to day 41 in the presence of 300 pg/ml exogenous TGF-β1, and then from day 41 without addition of TGF-β1. The other half were continued from day 21 without addition of TGF-β1. Data represent means±s.d. of four replicate cultures from a typical experiment.

output for a single plated cell was 10^8-10^9 in the control condition, and 10^{11}-10^{12} when 10 pg/ml TGF-β1 was added to the medium. Calculations made to cumulate the results from independent cultures indicated that this represents a mean increase from 28-29 to 37-38 PDs ($P<0.01$, $n=5$ experiments).

By contrast, 10 pg/ml TGF-β1 did not increase amplification of $Adh^{-/+}$ keratinocytes more than tenfold (from ~10^4 to ~10^5; Fig. 2A), which is about 1000-fold less than the effect observed on Adh^{+++} cells (from ~10^8 to ~10^{12}; Fig. 2A). These results suggest that this effect of TGF-β1 concerns mainly the most primitive cells. The slight increase in $Adh^{-/+}$ keratinocyte expansion might be due to the fact that the adhesion-based enrichment procedure was not effective in removing 100% of the Adh^{+++} cells from the $Adh^{-/+}$ population.

An inhibition of keratinocyte proliferation by TGF-β1 was observed in our culture system when high, but nevertheless physiological, concentrations (=100 pg/ml) were used (Fig. 2A,B). A significant reduction of the percentage of keratinocytes in S+G_2/M phase of the cell cycle was observed 24 hours after addition of TGF-β1 at concentrations of ⩾100 pg/ml (Fig. 3A). It is important to note that this growth-inhibitory effect appeared to be reversible. Indeed, keratinocytes initially cultured for 30 days with an inhibiting concentration of TGF-β1 started to divide as soon as the addition of the factor was stopped, and subsequently they did not show any reduced capacity to proliferate and expand (Fig. 3B). Moreover, the proliferation of keratinocytes initially cultured for 21 days without exogenous TGF-β1 could be transiently inhibited by TGF-β1 (⩾100 pg/ml) for 20 days, without any alteration of their subsequent long-term expansion potential (Fig. 3C). These observations confirm those of a previous study, showing that exposure for 48 hours to TGF-β1 mediates a reversible growth arrest and does not alter the clonogenic capacity of keratinocytes (Shipley et al., 1986), and suggest that TGF-β1 does not induce apoptosis of stem and progenitor cells, even at high physiological concentrations.

The promotion of epidermal precursor amplification by low TGF-β1 concentrations (10-30 pg/ml) was particularly impressive in cultures initiated with primitive $Adh^{+++}EGF\text{-}R^{low}$ cells. Indeed, in the experiment shown in Fig. 2B, the mean output for one $Adh^{+++}EGF\text{-}R^{low}$ plated cell was 10^{11}-10^{12} in the control condition and this value reached 10^{16}-10^{17} in the cultures treated with 10 pg/ml TGF-β1, representing an increase from 38-39 to 55-56 PDs. Given that the initial cloning efficiency of $Adh^{+++}EGFR^{low}$ keratinocytes is less than ~2%

(results not shown), and making the assumption that only clonogenic cells contribute to the total cell output, we estimated that each clonogenic cell in this subpopulation can generate more than ~10^{18} keratinocytes (~60 PDs). One feature of the most primitive epidermal cells is their smaller size in comparison with more mature keratinocytes committed to differentiation (Barrandon and Green, 1985). As shown in Fig. 2C, a low concentration of TGF-β1 not only increased the cumulative keratinocyte expansion, but also prolonged the production of clones composed of small, undifferentiated keratinocytes. It is also important to note that TGF-β1-mediated expansion did not alter the organogenic potential of epidermal precursor cells, which showed a continuous capacity to form an epidermis equivalent to that of cells cultured without a low TGF-β1 concentration (results not shown).

Effects of low TGF-β1 concentrations on keratinocytes at the molecular level

TGF-β intracellular signaling is mediated through Smad, the mammalian homolog of the *Drosophila* Mothers against dpp (Mad). This cascade involves Smad1, Smad2 and Smad3 as direct targets of the type I TGF-β serine/threonine kinase receptor family (Abdollah et al., 1997; Liu et al., 1997; Souchelnytskyi et al., 1997; Chen et al., 1999) (reviewed by Moustakas et al., 2001). To determine whether concentrations of TGF-β1 as low as 10 pg/ml are able to activate the Smad pathway, we next analyzed the degree of Smad1 and Smad2/3 phosphorylation in cultures of keratinocytes, initiated with $Adh^{+++}EGF\text{-}R^{-/+}$ cells, and grown with or without 10 pg/ml TGF-β1. Phosphorylated forms of Smads, p-Smad1 (Ser463/Ser465) and p-Smad2 and 3 (Ser433/Ser435), were quantified and compared in each culture condition (Fig. 4). Results indicate that a treatment with 10 pg/m TGF-β1 is sufficient to increase Smad phosphorylation. In the representative control experiment shown, 47.2% and 26.1% of the keratinocytes were detected as positive cells for the presence of p-Smad1 and p-Smad2/3 respectively. These percentages reached respectively 76.3% and 54.3% in the presence of 10 pg/ml TGF-β1. Similarly, the median values of p-Smad1 and p-Smad2/3 fluorescence were respectively increased from 5.2×10^6 and 3.2×10^6 arbitrary units (a.u.), in the control condition, to 7.6×10^6 and 5.8×10^6 a.u. in TGF-β1-supplemented culture.

A high expression of α6 (CD49f) and β1 (CD29) integrin chains is known to be associated with an immature state of keratinocytes (Jones et al., 1995; Li et al., 1998). To investigate further the promotion of long-term amplification of epidermal precursors by low TGF-β1 concentrations, we analyzed and compared the expression of these cell-surface markers in long-term cultures initiated with $Adh^{+++}EGF\text{-}R^{-/+}$ cells and performed with or without 10 pg/ml TGF-β1. Typical labeling distributions obtained from cultures at passage 4 are presented in Fig. 5 (A-F). In the control culture, 53.1% and 66.1% of the keratinocytes were detected as positive cells for the expression of α6 and β1 integrins respectively (Fig. 5C,D). These percentages reached respectively 92.4% and 83.4% in the presence of 10 pg/ml TGF-β1 (Fig. 5E,F). Similarly, the median values of α6 and β1 integrin fluorescence were respectively increased from 9.5×10^6 and 11.3×10^6 a.u. in the control condition (Fig. 5C,D) to 16.7×10^6 and 16.5×10^6 a.u. in TGF-β1-supplemented culture (Fig. 5E,F), indicating a higher degree of immaturity in this optimized culture condition.

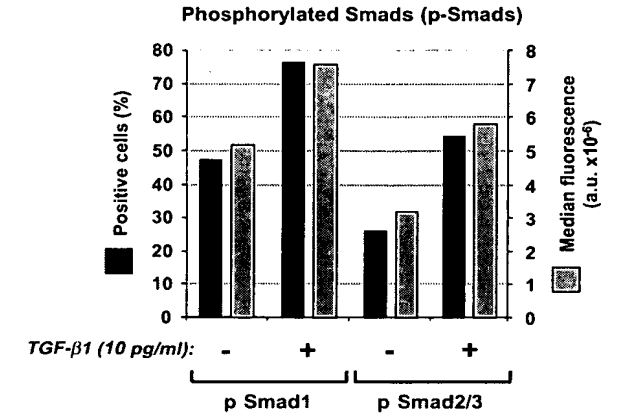

Fig. 4. Effect of a low TGF-β1 concentration on Smad1 and Smad2/3 phosphorylation. Keratinocytes from cultures initiated with $Adh^{+++}EGF\text{-}R^{-/+}$ cells were plated on glass slides and grown for 72 hours with or without 10 pg/ml TGF-β1. The degree of Smad1 and Smad2/3 phosphorylation (presence of p-Smad1 and p-Smad2 and 3) was then evaluated in each condition by immunofluorescence and compared by Laser Scanning Cytometry (LSC) analysis. Results from one representative experiment are presented (n=3). Data are expressed as percentages of positive cells and median values of fluorescence (arbitrary units, a.u.) detected in the two culture conditions.

Conclusions

We have developed a feeder layer-free culture system in which cell cycling of epidermal precursors can be transiently blocked by the addition of high TGF-β1 concentrations (≥100 pg/ml), and keratinocyte expansion can be stimulated by the addition of very low TGF-β1 concentrations (10-30 pg/ml).

The involvement of TGF-β1 signaling in the control of the homeostasis of the epidermis has been confirmed in vivo in various transgenic mouse models (Cui et al., 1995; Wang et al., 1997; Amendt et al., 1998), and suggested in humans by clinical observations showing that a dysregulation of the TGF-β1 signaling cascade is often associated with the malignant conversion of skin keratinocytes (Lange et al., 1999). However, although these in vivo observations confirmed the inhibition of keratinocyte cell cycling described in vitro with high concentrations of TGF-β1, they do not provide any information about the positive effect of TGF-β1 on immature keratinocyte expansion, as demonstrated in this in vitro study with very low concentrations (10-30 pg/ml).

The potent biphasic effect of TGF-β1 previously reported for the development of hematopoietic progenitor cells (Fortunel et al., 2000a) and osteoclast-like cells (Shinar and Rodan, 1990) for in vitro angiogenesis (Pepper et al., 1993) and mammary gland ductal morphogenesis (Soriano et al., 1996) is here clearly demonstrated for human skin precursor cells. Functional studies may be applied in such culture systems to elucidate further the complex regulatory network involved in the exquisitely dose-dependent response of cells to

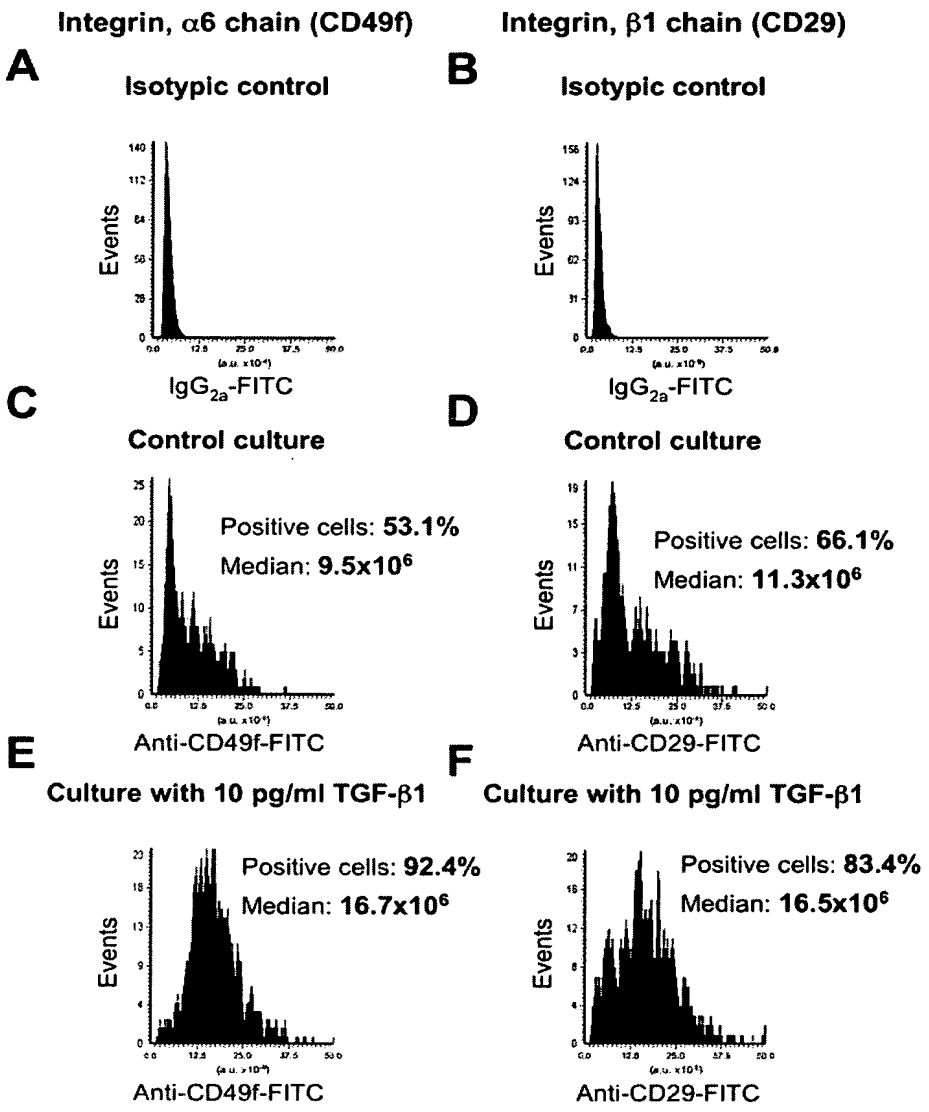

Fig. 5. Effect of a low TGF-β1 concentration on α6 (CD49f) and β1 (CD29) integrin expression by keratinocytes. Long-term cultures were initiated with $Adh^{+/+}EGF\text{-}R^{-/+}$ cells and performed with or without 10 pg/ml TGF-β1. Cells at passage 4 were plated on glass slides, grown for 72 hours, and then processed for immunofluorescence. Expression of integrins was analyzed by Laser Scanning Cytometry (LSC) and compared in the two culture conditions. (A) Isotypic control corresponding to α6 integrin labeling. (B) Isotypic control corresponding to β1 integrin labeling. (C) α6 integrin expression profile of keratinocytes grown in the control condition. (D) β1 integrin expression profile of keratinocytes grown in the control condition. (E) α6 integrin expression profile of keratinocytes grown in the presence of 10 pg/ml exogenous TGF-β1. (F) β1 integrin expression profile of keratinocytes grown in the presence of 10 pg/ml exogenous TGF-β1. Representative labeling profiles from a typical experiment are presented (n=3).

TGF-β1. In addition to the various effectors regulating TGF-β1-induced cell-cycle arrest in keratinocytes (Hannon and Beach, 1994; Ivarone and Massagué, 1999), it will be interesting to focus on genes whose expression may be linked to the maintenance of the epidermal stem cell pool, such as $p21^{(WAF1/Cip1)}$ (Topley et al., 1999), 14-3-3σ (Dellambra et al., 2000), P63 (Pellegrini et al., 2000), c-Myc (Waikel et al., 2001), β-catenin (Zhu and Watt, 1999; Huelsken et al., 2001), genes involved in delta-notch signaling (Lowell et al., 2000), and the transcriptional regulators Tcf3 and Lef1 (Merril et al., 2001).

We describe here the capacity of low TGF-β1 concentrations to promote long-term expansion of undifferentiated human epidermal precursors, an effect that was suggested, but not demonstrated, in the hematopoietic system. However, much remains to be done to characterize this function at the molecular level. In an avian system, it has been reported that TGF-β1 is capable of sustaining erythrocytic progenitor cell proliferation and self-renewal, and that this effect occurs through a cooperation between the TGF-β and TGF-α receptors via the Mek-Map kinase pathway (Gandrillon et al., 1999). In the human hematopoietic system, TGF-β1 could participate in the maintenance of a pool of primitive and undifferentiated progenitors in part by upmodulating the immaturity marker, CD34, on cycling cells (Batard et al., 2000; Pierelli et al., 2000). TGF-β1 has not yet been demonstrated to regulate directly the expression of the mucin-like protein CD34 in normal hematopoietic stem/progenitor cells, but this has been demonstrated in the pluripotent erythroleukemia cell line TF-1. Marone et al., have shown that TGF-β1 transcriptionally activates CD34 and then prevents differentiation of TF-1 cells, by acting independently through Smad, TAK1 and p38

pathways (Marone et al., 2002). The downstream component of the Wnt signaling cascade, β-catenin, appears to be an important molecule especially in epidermal stem cells (Zhu and Watt, 1999; Huelsken et al., 2001). Thus, as described in the early amphibian embryo during the formation of Spemann's organizer (Nishita et al., 2000), a possible interaction between Wnt and TGF-β signaling should be investigated in the case of epidermal precursors treated with the low TGF-β1 concentrations described here to promote expansion.

One important clinical application of this work is the possibility of improving the culture systems currently used to amplify keratinocytes for skin grafting (Ronfard et al., 2000) by the addition of the TGF-β1 concentrations described here to optimize the long-term expansion of immature keratinocytes. Furthermore, the general principle applied here in purifying primitive cell subpopulations could be used as an approach to purify precursor cells from other somatic tissues. Similarly, since the manipulation of TGF-β1 appears to be effective in permitting in vitro expansion of primitive epidermal cells, it would be of interest to investigate whether similar use and manipulation of TGF-β1 might be applied in the expansion of other adult tissue precursors.

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TGF-β1 maintains hematopoietic immaturity by a reversible negative control of cell cycle and induces CD34 antigen up-modulation

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SUMMARY

Somatic stem cells are largely quiescent in spite of their considerable proliferative potential. Transforming growth factor-β1 (TGF-β1) appears to be a good candidate for controlling this quiescence. Indeed, various mutations in the TGF-β signalling pathway are responsible for neoplasic proliferation of primitive stem/progenitor cells in human tissues of various origins. In hemopoietic single cell culture assays, blocking autocrine and endogeneous TGF-β1 triggers the cell cycling of high proliferative potential undifferenciated stem/progenitor cells. However, it has never been demonstrated whether TGF-β1 has an apoptotic effect or a differentiating effect on these primitive cells, as already described for more mature cells.

Using single cell experiments both in liquid or semi-solid culture assays and dye tracking experiments by flow cytometry, we demonstrate that low, physiological concentrations of TGF-β1, which specifically maintain primitive human hemopoietic stem/progenitor cells in quiescence, have a reversible effect and do not induce apoptosis. We moreover demonstrate that these low concentrations prevent the rapid loss of the mucin-like protein CD34, a most common marker of immature hematopoietic stem/progenitor cells, which is progressively lost during differentiation. TGF-β1 not only up-modulated the CD34 antigen before S phase entry but also maintained a high level of CD34 expression on cells which had escaped cell cycle inhibition, suggesting that proliferation inhibition and differentiation control by TGF-β1 may be independent. These data provide additional evidence that TGF-β1 acts as a key physiological factor ensuring the maintenance of a stem cell reserve.

Key words: TGF-β1, Quiescence, CD34 antigen, Progenitor immaturity, Hematopoiesis

INTRODUCTION

The past decade has been characterized by an increasing interest in stem cells because of their considerable therapeutic potential for gene transfer and long-term engraftment. In addition to the difficulty of characterizing stem cells, understanding the controls of their cell cycling, differentiation and possible apoptosis remains an important challenge. In this study, we have focused on the hematopoietic system which represents a good model for other types of somatic stem cells (Fortunel et al., 1998).

Normal adult hematopoietic stem/progenitor cells, as well as other somatic stem cells (Robinson et al., 1991; Potten et al., 1997; Puolakkainen et al., 1994), are generally quiescent or slowly cycling (Till and McCulloch, 1961; Till et al., 1964; Becker et al., 1965; Hodgson and Bradley, 1979; Suda et al., 1983). This characteristic distinguishes these cells from the more committed and differentiated progenitors or malignant progenitors the cell cycle status of which is more heterogeneous. The use of drugs which selectively kill cycling cells allows the selection of hematopoietic stem/progenitor cells (Berardi et al., 1995) by eliminating cycling differentiated and malignant cells.

This quiescent state may be due either to the absence of stimulating factors or to the presence of inhibitors. One well-known inhibitory cytokine is transforming growth factor-β1 (TGF-β1; Roberts et al., 1985; Massagué, 1990) which inhibits the early stages of normal cell development in both the murine (Ohta et al., 1987; Kishi et al., 1989; Keller et al., 1990, 1998; Ploemacher et al., 1993; Jacobsen et al., 1994, 1996) and human hematopoietic system (Ottman and Pelus, 1988; Sing et al., 1988; Hatzfeld et al., 1991; Sargiacomo et al., 1991; Jacobsen et al., 1991). In contrast, later stages are unaffected or even stimulated by TGF-β (Jacobsen et al., 1991; Keller et al., 1992; Hestdal et al., 1993; Turley et al., 1996). The first identified isoform, TGF-β1, is probably a physiological inhibitor since it is produced by stromal cells (Eaves et al., 1991). In addition, we have demonstrated by antisense experiments that early progenitors produce TGF-β1 and are also inhibited in an autocrine and/or a paracrine manner (Hatzfeld et al., 1991). However, these experiments do not address the question of whether the inhibitory effect of TGF-β1

is reversible or not. Indeed, these results could also be explained by an apoptotic effect of TGF-β1.

Reversibility has been suggested by resistance to 4-HC- or 5-FU-induced cytotoxicity (Lemoli et al., 1992; Grzegorzewski et al., 1994), and by delayed colony formation induced by TGF-β1 in batch culture (Sitnicka et al., 1996; Garbe et al., 1997). A protective effect has also been described with other cell systems and culture conditions (Dybedal et al., 1997). However, the reversibility of cell cycle inhibition by TGF-β1 has never been unambiguously demonstrated at a clonal level for normal early progenitors. Moreover, apoptosis has been suggested to be one possible mechanism of TGF-β1 inhibition (Lotem and Sachs, 1992; Taetle et al., 1993; Veiby et al., 1996).

In the present study, we have used two non-toxic methodologies, dye tracking and single cell sorting, to analyze, at a clonal level, the reversibility of quiescence induced by low concentrations of TGF-β1 which inhibit early high proliferative potential (HPP) progenitors. Not only were we able to demonstrate that TGF-β1 is not apoptotic and has a reversible effect on the progenitors which are both the most immature and the most sensitive to TGF-β1, but we also demonstrated that TGF-β1, at low concentrations, increases and maintains a high level of the CD34 mucin-like protein, the most common marker of immature progenitors (Civin et al., 1984; Berenson et al., 1988, 1991; Krause et al., 1996).

MATERIALS AND METHODS

Hematopoietic growth factors and antibody

Recombinant human (rh) IL3, rhIL6 and rhGM-CSF were generously provided by the Genetics Institute (Cambridge, MA). RhEpo, rhSF and rhG-CSF were supplied by Valbiotech (France), and rhTGF-β1 by R&D (UK). These cytokines were used, respectively, at 1.7 U/ml, 10 U/ml, 19 U/ml, 2 U/ml, 12 ng/ml, 3.3 U/ml, and from 30 or 3,000 pg/ml. A polyclonal TGF-β blocking antibody (anti-TGF-β) was raised in the chicken (StemBio Research, France) and used at 10 μg/ml. The anti-TGF-β neutralized the biological activity of rhTGF-β1 and also that of TGF-β2 but at a concentration 100 times higher. Ten μg/ml of antibody neutralize 1 to 3 ng/ml of rhTGF-β1 as tested in semi-solid cultures. Since the use of the anti-TGF-β antibody is reproduced by specific anti-TGF-β1 antisense (Hatzfeld et al., 1991), we consider that the effect of the antibody was mainly 'anti-TGF-β1'.

CD34⁺ cell preparation

Umbilical cord blood (UCB) samples were collected immediately after delivery using the method described by Brossard et al. (1990). Informed consent was obtained before sample collection. Early CD34⁺ cells were purified by MACS technique (Miltenyi Biotech, France) following the manufacturer's instructions. Since high purity was obtained (>95%), purified cells were considered as 'CD34⁺' cells.

Cell labelling and flow cytometry cell sorting

For single cell sorting, purified CD34⁺ cells were suspended in PBS/BSA (0.2%) and incubated with an anti-CD34 fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (MoAb) (clone 8G12; Becton Dickinson, San Jose, CA) for 30 minutes at 4°C and washed twice as previously described (Li et al., 1994). Irrelevant FITC-IgG1 Ab (Becton Dickinson) was used as negative control. The CD34⁺ cells were transferred into 96-well U-bottomed plates (Falcon) containing medium, using a Vantage fluorescence activated cell sorter (FACS; Becton Dickinson) equipped with an Automatic Cell Deposition Unit. One cell per well was deposited into the 60 central wells of the plates, the other wells being filled with water for better hydration. Five plates were prepared for each culture medium. This corresponds to a theoretical total of 300 cells isolated for each medium.

For dye tracking, CD34⁺ cells were labelled with PKH26 dye (Sigma, France) according to the manufacturer's instructions. A narrow peak of labelled cells was obtained by sorting cells with intensity (I_{cell}) in a region centered on the mean intensity (I_{mean}) of total labelled cells before sorting. We defined its minimal (I_{min}) and maximal (I_{max}) intensities ($I_{min} \leq I_{cell} \leq I_{max}$) so that the region width ($I_{width} = I_{max} - I_{min}$) represents about 10% of this mean intensity. This strict condition of sorting was defined to avoid the undesirable effect of widening of peaks, due to asymetric division and other biological or physical phenomena during culture. In such conditions, it was possible to discriminate peaks associated with each successive division.

The CD34⁺ cells thus sorted corresponded to about 20% of the total CD34⁺ population. A sample was cultured in semi-solid medium. The remaining cells were cultured in liquid medium for 3 days and analyzed again. The compartments of undivided cells or proliferating cells were sorted and cultured in semi-solid medium. Immunobrite beads (Coulter-Beckman, France) were used to standardize the FACS settings between day 0 and day 3.

Liquid cultures

For single cell studies (Fig. 1A), liquid SBA medium (StemBio Research, France) with 24% fetal calf serum was used. One hundred μl of medium containing IL3, IL6, SF, G-CSF, Epo and GM-CSF were distributed in wells at day 0. When necessary, TGF-β1 was added at 300 pg/ml. One hundred μl of medium with anti-TGF-β1 were added after 4 days. After 10 days, 100 μl (half a well) were transferred into flat 96-well plates and 100 μl of medium containing anti-TGF-β1 were added.

For dye tracking cultures (Fig. 1B), 30 to 50,000 PKH26-labelled and sorted cells were cultured in 1 ml of medium in 24-well plates (Falcon) in the presence of TGF-β1 or anti-TGF-β1 for 3 days. They were then harvested and counted in a Malassez chamber, analyzed, sorted by FACS and then recultured.

Semi-solid cultures

100-300 cells were cultured in semi-solid HPP-Q* medium (StemBio Research, France) containing IL3, IL6, SF, G-CSF, GM-CSF, Epo and anti-TGF-β1 (Fortunel et al., 1998).

Apoptosis detection

Purified CD34⁺ cells were cultured in liquid SBA medium with the above specified cytokines with or without TGF-β1 (30 or 300 pg/ml). Cells were harvested every 24 hours and treated with the annexin V FITC Kit (Immunotech, France) following the instructions of the manufacturer. The percentage of apoptotic cells was determined by flow cytometry (FCM) analysis.

Counting and microscopic analysis

For single cell studies, clones were analyzed in each well under an inverted Wilovert microscope a few hours after sorting, 3 days later and from 20 to 28 days later. In semi-solid cultures, the types of progenitor were determined from 14 to 18 days, as already described (Fauser and Messner, 1978, 1979; Zhou et al., 1988). In many cases, they were confirmed by staining with a RAL555 kit (Prolabo, France) after cytocentrifugation on a Cytospin centrifuge (Shandon Elliot, PA). HPP progenitors were defined as cells giving rise to clones larger than one quarter of the well in single cell experiments at day 20. This represents more than 100,000 cells for HPP-BFU-E. In semi-solid culture, HPP-BFU-E were defined as BFU-E with more than 10 clusters.

Calculations and statistics

In single cell experiments, each well, which initially contained one

cell, was evaluated for its day 3 cell number, its day 20 size index and the clone phenotype.

In dye tracking experiments, the percentage (p_k) of cells corresponding to k divisions ($0 \leq k \leq 5$) after 3 days of culture was defined using Modfit software. Since one $CD34^+$ which symetrically divides k times, produces 2^k day 3 cells, one day 3 cell which corresponds to k divisions, represents the contribution of $1/2^k$ day 0 $CD34^+$ cell. The percentage of day 0 $CD34^+$ ($p_k°$) cells, which produce cells divided k times, was therefore calculated as follows:

$$p_k° = \frac{\text{contribution of day 0 CD34}^+ \text{ to day 3 cells with k divisions}}{\text{total contribution of day 0 CD34}^+}$$

$$p_k° = [p_k/2^k]/\Sigma_{0 \leq j \leq 5}(p_j/2^j) .$$

The significance of results was analyzed by the paired Student's *t*-test.

RESULTS

In order to demonstrate the reversibility of cell cycle inhibition by TGF-β1 at a clonal level, our study was carried out in three steps: (1) analysis of the cell cycle status in control medium and correlation of quiescence or slow cycling with immaturity; (2) analysis of cell cycle inhibition by TGF-β1; (3) analysis of the reversibility of this inhibition.

Positive correlation between quiescence and early hematopoietic potentials

Our first aim was to compare the quiescent or proliferative status of progenitors with various potentials: high proliferative potential pluripotent erythro-myeloid (HPP-MIX), high proliferative potential burst forming unit-erythroid (HPP-BFU-E) and later BFU-E. This was performed using two different techniques: 'single cell sorting' and batch culture 'dye tracking' as shown in Fig. 1.

In single cell sorting experiments (Fig. 1A), $CD34^+$ cells were sorted in control medium (CM) with or without TGF-β1, at 1 cell per well, in 300 wells, individually checked by microscopic observation at day 0 (sorting efficiency >95%). After 3 days, the cell number in each well was evaluated again by microscopic observation. Then medium containing TGF-β1 blocking antibody (anti-TGF-β1) was added at 4 and 10 days to abrogate reversible TGF-β1-dependent effects. The type and size of each clone were analyzed 20 to 28 days later. Several informative parameters were obtained by this protocol: the percentages of potential HPP-MIX, HPP-BFU-E and BFU-E in cells which were $CD34^+$ on day 0, and for each of these categories, the percentage of undivided or dividing clones with their mean size after 3 days of culture. Clones which remained undivided at day 3 were considered as 'quiescent' and not dying if they exhibit clonogenicity within 28 days of culture.

In dye tracking batch culture experiments (Fig. 1B), $CD34^+$ cells were labelled with PKH26. A narrow peak of PKH26 labelled cells was sorted at day 0 to follow more accurately successive cell divisions in liquid CM. This procedure did not select particular progenitors (data not shown). After 3 days, the cells were analyzed by FCM and undivided progenitors were sorted according to their higher PKH26 fluorescence. The percentage and potential of day 0 progenitors which remained reversibly quiescent until 3 days were determined by comparing semi-solid culture of day 0 $CD34^+$ cells and of day 3 sorted undivided cells. Their clone phenotype was evaluated after 14 to 18 days.

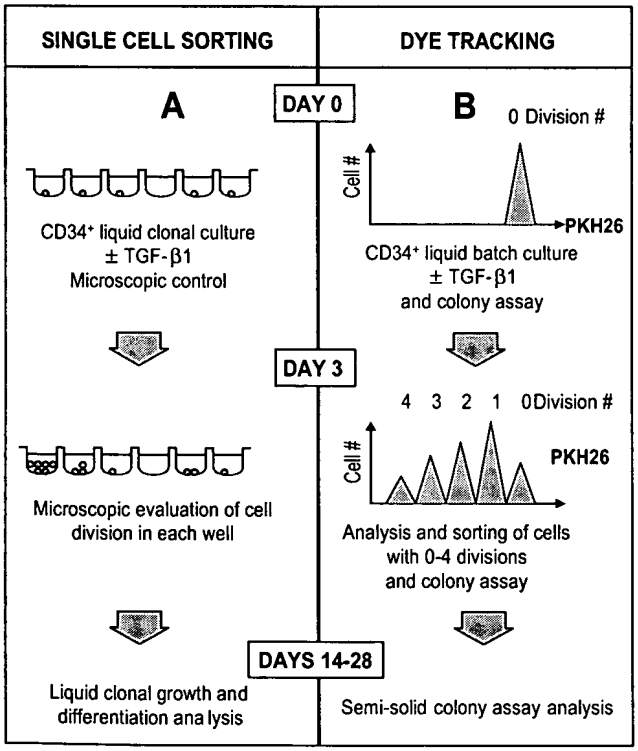

Fig. 1. Schematic diagram of assays.

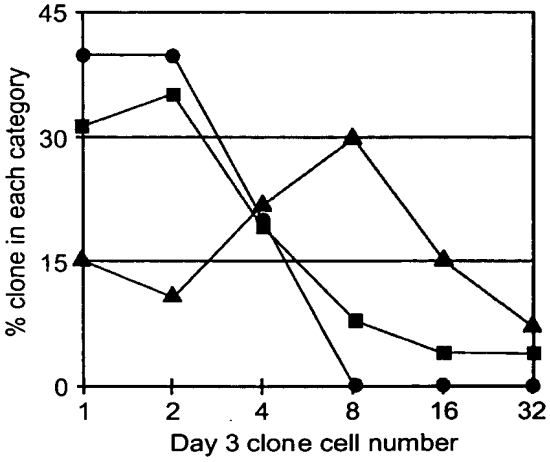

Fig. 2. Distribution of progenitors as a function of day 3 clone size. Three hundred $CD34^+$ cells were isolated by flow cytometry and cultured in control medium. After 3 days, clone size was evaluated by microscopic observation. This figure illustrates the results of one typical experiment (N=3). HPP-CFU-Mix (●), HPP-BFU-E (■), BFU-E (▲).

Table 1. Correlation between progenitor immaturity, day 3 quiescence and clone size

Progenitor type (% of CD34+ cells at day 0)*		% of day 3 quiescent cells‡	Mean number of cells per clone (day 3)
HPP-MIX (2.3)*	exp 1	40	2.0
	exp 2	75	1.3
	Mean	58	1.7
HPP-BFU-E (8.7)*	exp 1	31	3.5
	exp 2	62	1.7
	Mean	47	2.6
BFU-E (11)*	exp 1	15	7.1
	exp 2	14	4.9
	Mean	15	6.0

‡The percentage of quiescent cells represents the fraction of progenitors of each category which remains undivided after 3 days but generates colonies after longer culture.

Results clearly show an association of immaturity with quiescence or slow cycling. HPP-Mix were more quiescent than HPP-BFU-E, which were themselves more quiescent and slow cycling than BFU-E. This is well-illustrated for each progenitor type by clone size distribution after a 3-day culture in Fig. 2. The results obtained for single cell sorting are detailed in Table 1 and showed that: (1) HPP-MIX, HPP-BFU-E and BFU-E represent, respectively, 2.3%, 8.7% and 11% of $CD34^+$ at day 0; (2) more HPP-MIX remain quiescent at day 3 (58%) than HPP-BFU-E (47%) and than BFU-E (15%); (3) the mean size of HPP-MIX clones (1.7 cells/well) at day 3 was lower than that of HPP-BFU-E (2.6 cells/well) or BFU-E (6.0 cells/well). A similar positive correlation between immaturity, quiescence and slow cycling status was also observed in 3 different experiments by dye tracking (data not shown). All these results demonstrate a significant correlation between quiescence and immaturity.

Analysis of cell cycle inhibition by TGF-β1

TGF-β1 is well-known to inhibit clonogenic growth or proliferation of early hematopoietic progenitors. However, this effect is lineage- and dose-dependent. In particular, previous results from our laboratory have shown that the 50% inhibitory dose (ID_{50}) is as low as 10 pg/ml of TGF-β1 for HPP-MIX and increases with differentiation status (e.g. 100 pg/ml for early erythroid HPP-BFU-E and >1,000 pg/ml for later BFU-E; Fortunel et al., 1998, personal results). We chose 300 pg/ml as it is both a concentration which can be found in human plasma in an active form (Junker et al., 1996) and which inhibits selectively the more primitive HPP-Mix and -GM, as well as HPP-BFU-E.

A well-defined population of PKH26 labelled $CD34^+$ cells was sorted and transiently incubated in culture medium with or without TGF-β1 or anti-TGF-β1. TGF-β1 was added at 300 pg/ml to selectively inhibit the more immature progenitors. After 3 days, the total cell population was analyzed to evaluate the quiescent subpopulations. Since $CD34^+$ purity was 98.2±1.1 (mean ± s.d. of 3 independent experiments) before culture and cells remain $CD34^+$ during a 3-day culture (see Fig. 5), these analyses concern exclusively $CD34^+$ cells. Typical flow cytometric analysis is shown in Fig. 3. TGF-β1

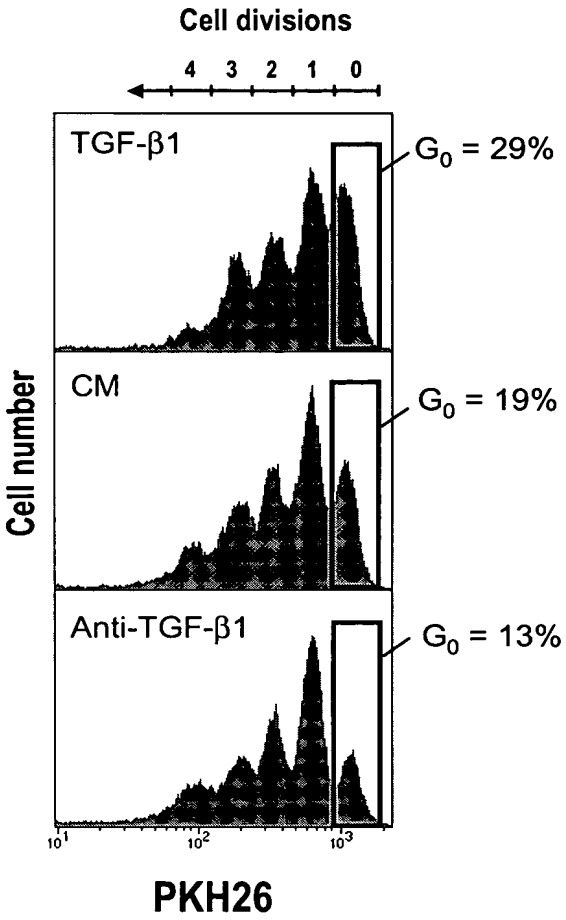

Fig. 3. Effects of TGF-β1 and anti-TGF-β1 on cell proliferation and quiescence. This is a representative example of a histogram obtained with PKH26 labelled $CD34^+$ cells at day 3. A narrow peak of PKH26 labelled $CD34^+$ cells was sorted at day 0 and cultured with or without TGF-β1 and anti-TGF-β1. PKH26 labelling was analyzed 3 days later.

significantly increased the undivided fraction on day 3 in comparison with control medium. Conversely, anti-TGF-β1 decreased this fraction. These quiescent fractions contained 24±6.2%, 35±5.7% and 42±4.3% (mean ± s.d. of 3 independent experiments) of the total $CD34^+$ progenitors in the anti-TGF-β1, control and TGF-β1 culture conditions, respectively.

These TGF-β1 or anti-TGF-β1 effects on cell cycle were similarly observed in single cell liquid or semi-solid experiments (data not shown).

Is TGF-β1 an inducer of apoptosis or a reversible cell cycle inhibitor?

The third step of our study was then to analyze whether inhibition is reversible or associated with apoptosis. If the hypothesis that TGF-β1 inhibits proliferation by the induction of apoptosis is true, then we should observe a loss of colonies after exposure to TGF-β1 and its subsequent neutralization, in

Table 2. Viable clones after 3 days with or without TGF-β1 treatment in single cell culture

	Clone phenotype		
Culture medium	Erythro-myeloid	Erythroid	Total
CM	7.0±4.2	60±17	224±4.2
CM+TGF-β1	6.5±3.5	62±2.8	221±0.7

Three hundred $CD34^+$ cells isolated by FACS were cultured with or without 300 pg/ml TGF-β1. Viable clones were determined by microscopic observation 3 days later. Results are the mean ± s.d. of 2 independent experiments. Anti-TGF-β1 was then added to abrogate reversible TGF-β1 effects. Clone phenotypes were determined 2 to 3 weeks later.

comparison with control medium. However, the absolute numbers of viable clones (CFU-MIX, total BFU-E or total clones) with or without TGF-β1 pretreatment were not significantly different in single cell experiments as shown in Table 2. In addition, TGF-β1 did not induce a significant loss of clonogenicity of day 3 undivided progenitors in tracking experiments (57±6 for TGF-β1 versus 64±13 for control medium; mean ± s.d. of 3 independent experiments; P=0.44). Moreover, in experiments where apoptosis was analyzed by FCM using Annexin-V-staining, we observed no more than 1.4±0.5%, 1.3±0.9% and 1.6±1.0% apoptotic cells (mean ± s.d. of 3 independent experiments) after 3 days of culture with respectively 0, 30 and 300 pg/ml TGF-β1.

Together these results demonstrate that, in both culture systems, TGF-β1 negatively but reversibly controls the cell cycle of hematopoietic progenitors without inducing detectable cell death.

Expression of CD34 antigen during proliferation and its modulation by TGF-β1

We have observed that low concentrations of TGF-β1 reversibly inhibited cell cycling of early clonogenic progenitors. Previous studies have shown that the immaturity-associated CD34 antigen disappears rapidly during cell proliferation. Indeed, proliferative media are most often differentiating media. We wanted therefore to study the possibility that the maintenance of a slow cycling state by TGF-β1 could be associated with the maintenance of an undifferentiated state and, particularly, with the maintenance of a high level of CD34 antigen.

CD34 expression was first analyzed during the first 19 hours, before cell division occurs. The effect of TGF-β1, at different concentrations, was then determined. Fig. 4 shows a TGF-β1-dependent increase in CD34 intensity up to 100 pg/ml where a plateau was reached.

To follow the modulation by TGF-β1 of CD34 expression during proliferation, purified $CD34^+$ cells were labelled with PKH26 and a narrow, but representative, peak of PKH26 labelled cells was sorted and cultured for 3 days. Cells were then labelled with FITC-conjugated CD34 antibody and analyzed. Results show that CD34 expression decreased progressively with proliferation, particularly in anti-TGF-β1 medium (Fig. 5). Low concentrations of TGF-β1 were sufficient to induce the appearance of a subpopulation which still expressed a high level of CD34 antigen after 2 to 4 divisions (Fig. 5; 14.2±4.4% for TGF-β1 versus 3.5±2.2% for anti-TGF-β1; mean ± s.d. of 3 independent experiments). These results demonstrate for the first time the ability of

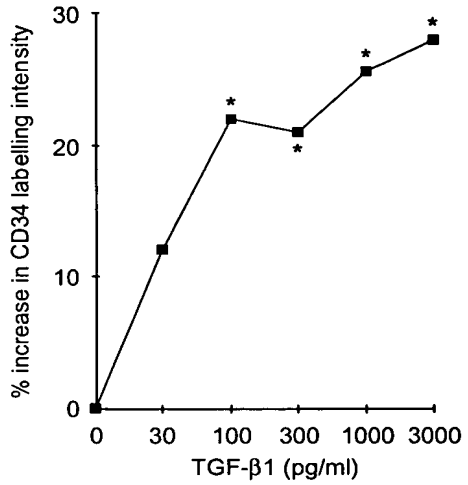

Fig. 4. Effects of increasing concentrations of TGF-β1 on CD34 antigen level at 19 hours. $CD34^+$ cells were cultured with increasing concentrations of TGF-β1 for 19 hours. Cells were then labelled with PE-conjugated CD34 antibodies and analyzed by flow cytometry in 3 independent experiments. The increase in mean intensity in comparison with the control medium was then calculated for each TGF-β1 concentration. Significance (*P<.05) was determined by the paired Student's t-test in comparison with CM.

TGF-β1 to maintain or up-modulate the expression of a membrane antigen associated with the hematopoietic stem/progenitor cell undifferentiated phenotype.

DISCUSSION

In this study, we have demonstrated that the more primitive umbilical cord blood $CD34^+$ cells are preferentially maintained in quiescence or in a slow cycling state by low TGF-β1 concentrations. Single cell experiments demonstrated that this effect is reversible and not related to apoptosis. Most interestingly, TGF-β1 promotes the maintenance of CD34 mucin-like protein expression, suggesting that this effect may prevent differentiation during early cycling and therefore maintain the stem cell compartment.

Although a reversible effect of TGF-β1 on quiescence was suggested by previous studies, including ours, it has never been clearly demonstrated that this was not due to an apoptotic effect of TGF-β1. This is here clearly demonstrated by single cell experiments, using human primitive hematopoietic cells able to produce clones of more than 10^5 cells both in liquid and semi-solid media. This demonstration was necessary as the question of a possible apoptotic effect of TGF-β on these primitive cells is still frequently raised (Lotem and Sachs, 1992; Taetle et al., 1993; Veiby et al., 1996).

In this study, we have mostly used a concentration of 300 pg/ml of TGF-β1. We have measured by annexin FCM experiments the effect of TGF-β1 concentrations between 30 pg and 300 pg, without detecting any selective apoptotic effect on the more primitive cells.

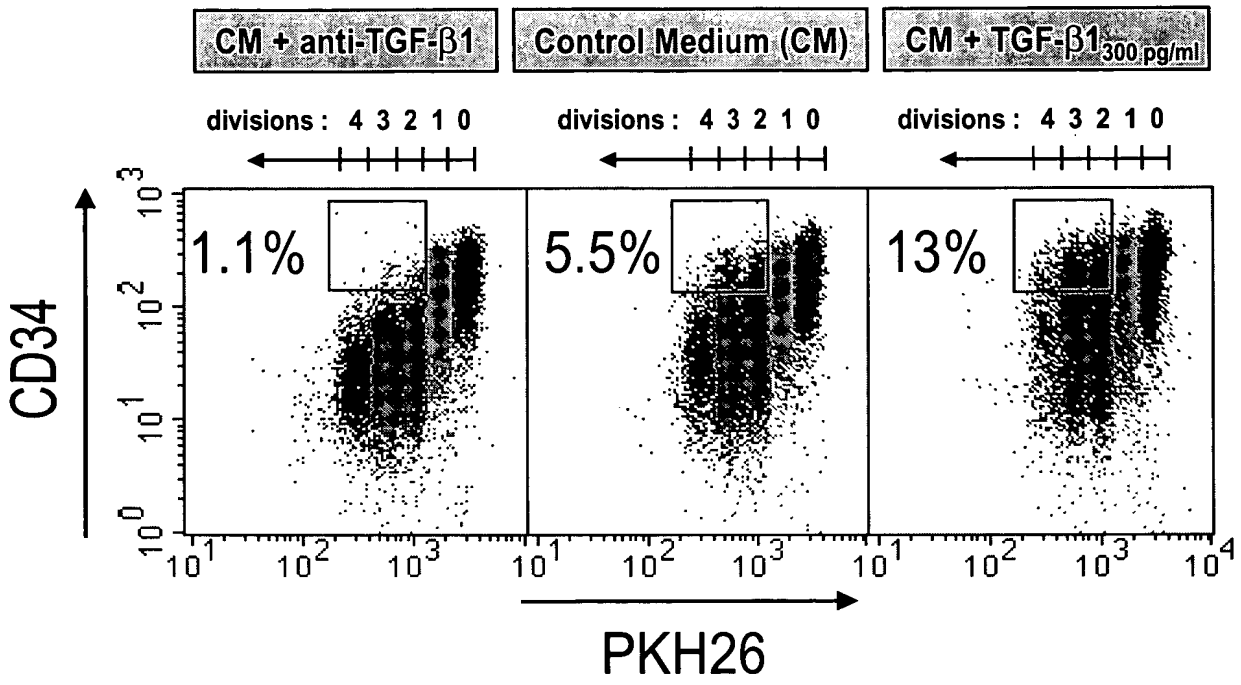

Fig. 5. Expression of CD34 antigen during cell proliferation and modulation by TGF-β1 and anti-TGF-β1. A narrow peak of PKH26 labelled CD34$^+$ cells was sorted at day 0 and cultured with or without TGF-β1 and anti-TGF-β1. Cells were labelled with FITC-conjugated antibodies 3 days later and simultaneous CD34/PKH26 labelling was analyzed. The analysis of the 3 culture conditions in one representative experiment out of 3 is shown.

This is not surprising as the TGF-β-induced cell cycle block occurs in G_1, around the theoretical restriction point (Ando and Griffin, 1995), before S phase entry, at the safest stage of the cell cycle to return and remain in G_0 for a few days, some months or many years.

In our previous studies, both autocrine TGF-β1 induction by retroviral supernatant, or tyrosine kinase receptor down-modulation in response to TGF-β1, were rapid and transient (Sansilvestri et al., 1995), suggesting that once a cell has returned to G_0, it does not require a continuous production of TGF-β1 to remain quiescent. However, different levels of quiescence may exist. The more primitive subpopulations of the stem cell compartment may be sensitive to concentrations not higher than 10 pg of active TGF-β1 which could be found in plasma or in the bone marrow microenvironment.

It should be noted that the active TGF-β1 concentration we have chosen in our assays corresponds to the upper range of active TGF-β1 levels found in the plasma of healthy donors (100±200 pg/ml) (Junker et al., 1996). These low TGF-β1 concentrations are sufficient to control most types of early human stem/progenitor cells, as we have demonstrated that the more primitive HPP-Mix respond to 10-30 pg/ml TGF-β1 and early HPP-GM and HPP-BFU-E respond to 30-300 pg/ml TGF-β1 (unpublished results, 1998). The bimodal TGF-β1 dose-response we observed in Fig. 4 with a significantly reduced response for concentrations higher than 100 pg/ml is reminiscent of results of many other studies in our laboratory in which we distinguished the effect of low 'physiological' TGF-β1 concentrations on cell cycle arrest of primitive cells, from higher concentrations which have a differentiation effect on late progenitors (Jacobsen et al., 1991; Keller et al., 1992; Hestdal et al., 1993; Turley et al., 1996). These higher concentrations have not been detected in the plasma. The possibility of locally higher concentrations cannot be excluded but should be under strict tissue-specific control (Rifkin et al., 1999), in view of the numerous other physiological effects of TGF-β1 (Roberts et al., 1985; Massagué, 1990).

Interestingly, our results demonstrate an up-modulation of CD34 antigen by TGF-β1. We have checked that this effect is not due to different FCM forward and side scatter of the cells treated with or without TGF-β1 (data not shown). Until now, we and others have demonstrated that cytokine receptors (KIT, FLT3, IL6-R, Trf-R) were down-modulated by TGF-β1 at both mRNA and protein levels (Sansilvestri et al., 1995; Fortunel et al., 1998). This is the first report, to our knowledge, of up-modulation of a membrane antigen by TGF-β1 in normal hematopoietic early progenitors. The fact that this antigen is the most common antigen associated with the earliest hematopoietic stages is of great interest. It might play a role in cytoadhesion in connection with the hematopoietic stroma and control both cell cycle inhibition and cell immaturity (Verfaillie et al., 1998). The fact that CD34 is highly expressed in dividing fetal liver stem cells suggests that the control of differentiation and quiescence by TGF-β1 might be independent. Understanding these controls is indispensible for the in vitro monitoring of self renewal of primitive stem progenitor cells

for cell therapy. The complex regulation by TGF-β of both proliferation and differentiation has been analyzed also in the epithelial system (Miettinen et al., 1994; Caulin et al., 1995). In the liver stem cell compartment, oval cells also express the CD34 antigen (Petersen et al., 1999).

The fact that TGF-β1 could control in vitro two important properties of adult stem/progenitor cells, quiescence and immaturity, is in agreement with in vivo experiments and clinical observations. We have previously shown that TGF-β1 is able to inhibit the growth of a subpopulation of primitive hematopoietic stem/progenitors which we call high proliferative potential-quiescent cells (HPP-Q) (Fortunel et al., 1998). The HPP-Q cells express low levels of various mitogenic receptors such as the tyrosine kinase receptors KIT/CD117, FLT3/CD135 and the IL6 receptor. These cells do not grow in a classical short-term culture system. In the rapid HPP-Q assay with added anti-TGF-β1, they give rise to large clones which can easily be counted. Interestingly, when KIT^{high}, KIT^{low}, or KIT^{-} cells are sorted, only the KIT^{low} cells which belong to the HPP-Q subpopulation provide a long-term engraftment when injected in a sheep foetus. KIT^{high} and KIT^{-} cells do not engraft (Kawashima et al., 1996).

Another argument to emphasize the importance of TGF-β1 in controlling the stem cell compartment is the growing number of clinical disorders involving hyperproliferation of primitive cells due to a mutation of one of the TGF-β RI or RII receptor genes or of the Smad genes. These cells produce TGF-β1 but cannot respond to it. They proliferate while inhibiting normal early stem/progenitor cells which respond to the TGF-β1 they produce (Le Bousse-Kerdiles et al., 1996; DeCoteau et al., 1997). These clinical disorders provide natural knock-out gene experiments in the human system. They clearly emphasize the important role of TGF-β1 in the control of the human hematopoietic stem cell compartment.

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